FRANÇOIS MAGENDIE

1783–1855

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(October 6, 1783–October 7, 1855)

François Magendie is more easily understood when one considers his life against the background of industrial, political and intellectual upheaval which characterized his era. With the rapid growth of industrial potential during the 18th century came measurable wealth to an ever increasing number of people. Such material gains, together with increased facilities for travel and communication, brought cultural advantages and with them a sense of importance hitherto reserved for the few. Inevitably the common people came to think of themselves as possessing at least some inalienable rights which they felt could be best served by a government composed of common people. Thus in America and in France republics were established, the French carrying their enthusiam so far as to chop off a king's head.

Once having been initiated, change brought forth more change, until it was the rule rather than the exception. The transcendental, the mystical, the dogmatic — pervading all thought and activity for centuries — were now suspect; indeed no aspect of human thought and endeavor was left unchallenged. Material gains spawned a materialistic philosophy.

It was inevitable that scientific thought should change also. No longer was it considered profitable to derive natural laws from dogmatically established postulates. The new fashion in scientific thinking was set by Auguste Comte's positivistic approach, which arrived at natural laws by inductive reasoning from carefully observed facts. The rediscovery of this method clearly defined the pattern of thinking which we have come to call the scientific method. Magendie himself was

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to say in his textbook, the Précis: "My principal object, in composing the following work, has been to contribute to the introduction of the Baconian method of induction into physiological science; at least I have done my best to present the science under the theoretical form; following meanwhile, in the exposition of facts, the inductive or analytical method." [1] In a sense it is amusing to follow the whole-hearted devotion of Magendie and others after him to the absoluteness of their modern science. Boruttau has said with grudgingly nationalistic overtones, "Der Ruhm, dem physiologischen Experiment wieder zu der Bedeutung, welche ihm Harvey und Haller einst gegeben hatten, verholfen und es auf die Grundlage absoluter 'Voraussetzungslosigkeit' gestellt zu haben, gebührt zwei Nichtdeutschen, in erster Linie wohl dem Franzosen Magendie." [2] There seems to have been little appreciation of the fact that the new approach simply substitutes one set of postulates for another, since it implies the validity of things reported by our senses.

With the new objective approach to all things scientific came the first great stirrings in chemistry and physics. These in turn were reflected in the developments made in the medical sciences, particularly in physiology and pathology where their applications were more directly apparent. With the material and cultural growth of the new middle class came a greater consciousness of the dignity of man and, for better or worse, a greater interest in his physical instead of his spiritual well-being. This shift in emphasis laid stress on improving sanitary conditions, controlling epidemics, and diagnosing and treating the diseases which afflict mankind. This in turn proved to be another impetus to the advances in medical sciences.

[1] Magendie, F. Précis eléméntaire de Physiologie. 1st ed., 1816. From translation by E. Milligan, An Elementary Compendium of Physiology for the Use of the Student. J. Carfrae and Son, Edinburgh, 4th ed., 1831.

[2] Boruttau, H. In M. Neuberger and J. Pagel's Handbuch der Geschichte der Medizin. Gustav Fischer, Jena, vol. 2, 1903.

Perhaps only in this atmosphere of change and uncertainty could a man like Magendie develop and thrive. In any other he might have been only a recalcitrant misfit.

François Magendie was born in Bordeaux October 6, 1783, the son of a successful surgeon. His schooling began when he was 10 years old and insisted on being sent to school. The liberal father, following the teachings of Rousseau, had permitted his children to do exactly as they pleased. In Magendie's case the system seems to have worked satisfactorily, since he soon distinguished himself in his studies. These were the days of insurrection; the strains of The Marseillaise scarcely muted the crashing of the guillotine and the rolling of title and untitled heads. Although the elder Magendie was a minor political figure of the new Republic, he was not immune from political persecution and was imprisoned for a short time. The effects of these affairs on the young Magendie are easily imagined. Nothing was stable, nothing permanent, nothing above suspicion.

Through the influence of the elder Magendie, François began his medical education at the age of 16. In 1793 the Commune had abolished the existing medical (and other) faculties; it had, in effect, virtually destroyed the universities. By decree education was free to anyone. Regrettably, there were few left to do the educating. The dangers of the chaotic medical training provided under the Commune were soon recognized, and remedied by the re-establishment of the medical schools of Paris, Montpellier and Strasbourg. Young Magendie thus came under the tutelage of the surgeon Boyer, member of the faculty of the new Paris school and of the staff of the Hôtel-Dieu and the Charité. The brilliant young man soon became prosector and began unofficially to give lectures in anatomy at the Charité. Dessault had just died, but his greatest pupil, Bichat, was continuing the famous lecture series at the Hôtel-Dieu. Although Bichat and Magendie were prevented from becoming rivals by the former's untimely death in 1802 Magendie felt compelled to combat for many years the doctrines which Bichat had in-

corporated in his famous "Traité des membranes" and "Anatomie générale appliquée à la physiologie et à la médecine." Bichat's teachings mark the transition from the transcendental to the materialistic philosophy in science; but his retention of vitalistic concepts aroused the ire of Magendie who, with his liberal background, had made almost the complete transition. A contemporary who was to offer real competition to Magendie's professional advancement was the surgeon Dupuytren, whose rise to prominence under Boyer rivaled and even excelled that of Magendie. The latter, however, was in no great hurry to attain prominence. Conscious of the defects of his own education, he supplemented his medical studies with courses in the classics and with independent work in comparative anatomy. In this subject he appears to have had the help and encouragement of Cuvier, who was to be a benign influence for some years.

Magendie, having begun his medical education under the informal system of the Revolution, was to complete his training under the rigid, "practical" policies of Napoleon. He completed his interneship, passed the required series of examinations and wrote his thesis on the uses of the soft palate. The certificate was awarded on March 24, 1808.

Supporting himself with the income from his position as aide in anatomy at the Faculty of Medicine and fees for his private lectures in anatomy, Magendie refrained for some time from taking up the active practice of medicine. He attended faithfully the meetings of the Paris Academy of Sciences and thus came under the influence of Laplace, who had collaborated with Lavoisier in studies on animal respiration, and of Lamarck, Berthollet, Gay-Lussac and Pinel, all of whom were present, according to Olmsted,[3] when Magendie

[3] Olmsted, J. M. D. François Magendie. Henry Schuman, Inc., New York, N. Y., 1944. Other sources of information are the following: Bing, F. C. Science, *74:* 456, 1931; Castiglioni, A. A History of Medicine. Alfred A. Knopf, New York, N. Y., 1941; Fulton, J. F. Selected Readings in the History of Physiology. Charles C. Thomas, Publ., Springfield, Ill., 1930; Garrison, F. H. An Introduction to the History of Medicine. W. B. Saunders Co., Philadelphia. Penna., 1929.

read his first paper before the Academy on April 24, 1809. Magendie had already attracted attention when he published in the Bulletin de la Société Médicale d'Émulation de Paris a paper entitled "Quelques idées générales sur les phénomènes particuliers aux corps vivan." In this he strongly attacked the vitalistic concepts of Bichat. The memoir read before the Academy of Sciences outlined clearly the methods of attack which Magendie employed in all his serious investigations and which became the cornerstone of experimental physiology. Its subject was the chemical and physiological properties of some vegetable poisons of the strychnos group. Employing surgical procedures of the type now common in experimental physiology and related sciences, Magendie studied the route of absorption of the drug and prepared a second paper for the Academy. In this he refuted the old concept that the lymphatics were the principal avenues of absorption. The publication of these papers established Magendie as a scientist of note, since they were mentioned in Cuvier's yearly summary of important scientific developments as well as in the textbooks being written at that time.

The next few years of Magendie's life were relatively uneventful from the scientific standpoint, no major work appearing in print. Suddenly in 1813 he announced a course of private lectures in experimental physiology and at the same time resigned his position in the anatomical laboratory. The reasons for his dramatic shift from surgery, for which he had been intensively trained, have been widely debated. Political pressure on the part of Dupuytren has been mentioned, as has Magendie's desire to make a name for himself in a new field in which competition was not as keen as in surgery. Olmsted [4] has still another idea: "It seems to have occurred to no one that Magendie may have renounced surgery because experimental physiology had captured his imagination." At this critical period in his development Magendie just barely escaped being called for army duty by a government which apparently cared only a little less about

[4] See footnote 3, page 6.

its scientific strength than is the custom today. Other than this, the momentous events of the time, the occupation of Paris, the Hundred Days, Waterloo, seem to have left him relatively undisturbed in the work of preparing his textbook, the "Précis élémentaire de Physiologie," whose first volume appeared in 1816.

The Précis not only enhanced Magendie's reputation but furnished a convenient vehicle for the publication of his own recent investigations. Soon learned societies in Sweden, Denmark, England, America and Germany placed him on their rolls, but the Paris Academy of Sciences did not yet see fit to honor him with membership. The advent of many new drugs led Magendie to compile his "Formulaire," a condensed account of their discovery, preparation, pharmacological properties and proper therapeutic use. In 1813 he had been elected to the Société philomathique and in 1820 became president of the Société Médicale d'Émulation de Paris, as well as co-editor of the Society's journal. In the following year he embarked upon the great adventure of founding his own journal, the "Journal de physiologie expérimentale," the first publication to be devoted exclusively to the new science which Magendie himself had established as an independent discipline. Honors followed quickly now; in 1821 he was elected to the newly created Royal Academy of Medicine and Surgery. In the same year he was elected to the Academy of Sciences, which had on several previous occasions considered him for membership but rejected him in favor of others nearly unknown today. There are indications that Magendie sought this honor above all others. He achieved his goal in the 38th year of his life. Perhaps the academicians were less than pleased with their choice when Magendie displayed during the sessions the rudeness and violent temper of which he was capable in scientific matters. Even the disappointment of the following year did not appreciably dampen his spirits. Laennec, inventor of the stethoscope, was appointed to the chair of medicine at the Collège de France.

With the publication of the work on direction of conduction in spinal roots and the subsequent controversy with Bell, Magendie appeared to have reached the pinnacle. He was not only venerated for his accomplishments but also vilified by antivivisectionists, particularly in England following a trip to that country. Further honors in the form of new appointments were now slow in coming. Not until 1826 was he appointed substitute physician at the Salpetrière. When the chair of medicine at the Collège de France fell vacant on the death of Laennec, Magendie's liberal ideas in politics and his uncompromising attitude in scientific matters caused him again to be passed by when the new appointment was made. It seemed to have been small comfort to him that the Academy of Sciences had almost unanimously backed him for the position, their recommendations being usually accepted by the king and his ministers. In his scientific reports he was perhaps growing careless or overconfident, or perhaps he had simply become too prominent. At any rate, a number of his publications were vigorously attacked by other workers. It must be admitted that he exhibited at this stage a tendency to draw rather sweeping conclusions from extremely limited data. This fault appears in some of his earlier work but had been largely ignored. By 1830 his fortunes seemed to have improved somewhat. Not only did he marry a widow of some means, but the revolution of that year brought an enforced vacancy of the chair of medicine at the Collège de France. Magendie was appointed without delay and was also made physician at the Hôtel-Dieu.

From this point on Magendie's efforts were principally those of a physician valiantly fighting epidemics (he did not consider cholera to be contagious), of an academician busy passing judgment on the work of others, and of a country gentleman. He did collaborate with Poiseuille in work employing the latter's mercury manometer for the measurement of blood pressure. In 1841 Claude Bernard was appointed to assist Magendie in the preparation of the demonstration experiments which invariably accompanied his lectures in

physiology. Bernard, who for several years previous had been unofficial assistant, soon busied himself with the task of drawing together the loose threads which Magendie often left dangling. The latter seemed never disturbed when the results of his experiments were at variance with those of other workers or when he himself obtained contradictory results. Bernard's was a much more orderly mind which would not tolerate inconsistencies and which was forced to search out the underlying causes of the contradictions. After collaboration on some of Mangendie's pet projects, Bernard, who received his medical degree in 1844, branched out largely on his own but apparently continued to receive encouragement from Magendie, with whom he remained on the friendliest terms.

Magendie resigned his official duties at the hospital in 1845 and became more than ever the country squire. Olmsted [5] paints a charming picture of the old gentleman entertaining his students at his country home at Sannois, operating a free clinic for the poor of the neighborhood, or puttering about the small laboratory he had established in his home. During his remaining years he argued vigorously against the introduction of anesthesia for surgical purposes. So influential had he become that the quarantine laws were considerably liberalized on his insistence that only typhus could be considered to be a contagious disease. Only some years after his death were these laws once more put into force. Magendie's last lectures at the Collège de France were given in the winter of 1851–1852. His health was rapidly failing and, having bequeathed his professorship to Bernard, he died October 7, 1855, at the age of 72.

Magendie's greatest contribution, aside from the reintroduction of the scientific method in physiology, was his work on the direction of conduction in spinal nerve roots published in 1822. Without doubt these efforts were stimulated by a visit from John Shaw of the Great Windmill Street School of Anatomy. Shaw had acquainted Magendie with the teach-

[5] See footnote 3, page 6.

ings of Charles Bell. The incisive demonstration by Magendie that the anterior spinal roots are concerned with motor activity and the posterior roots with sensory phenomena attracted immediate attention. Bell (and his associates) then claimed they had made this demonstration as early as 1811. To be sure, Bell's pamphlet of 1811 makes the claim that the anterior and posterior roots had different functions but seems to have been mistaken in what these functions were. He had, however, as Magendie expressed it, the germ of an idea. Bell's book of 1824 lays firm claim to priority but supports its argument with passages taken from older publications which had, however, been rewritten in the light of the newer developments. The controversy still rages today and is not resolved by the usual practice of speaking of the *Bell-Magendie Law.*

Less spectacular, perhaps, were Magendie's contributions to experimental pharmacology, a science he is often credited with having founded with his first scientific publication (on strychine, 1809). Later papers dealt with the action of emetine, morphine, iodides and bromides, among other substances. His "Formulaire" was a boon to the medical students of his day.

Magendie made major contributions to our knowledge of the alimentary tract, beginning with his dissertation on the soft palate and continuing with papers dealing with the esophagus and the process of regurgitation.

It is with Magendie's contributions in the field of nutrition that we are particularly concerned here. A memoir of 1816 on food substances without nitrogen is substantially included in the Précis. Discussing the various classes of food substances which had been proposed, Magendie offered a simpler breakdown into two categories: nitrogenous and nonnitrogenous. He then went on to argue that since tissues contain nitrogen and there is nitrogen present in foods, it seemed likely that tissue nitrogen was derived from foods. Others were arguing that tissue nitrogen was derived from the air, and based their theory primarily on the fact that certain

peoples subsisted on maize or rice (which were not supposed to contain nitrogen). To this Magendie replied " . . . and with regard to the people, as they say, who feed upon rice or maize, it is well known that they add milk or cheese; now casein is the most azotized of all the nutritive proximate principles." He also maintained that most foods contained some nitrogen. Recognizing the need for experimenting with nitrogen-free diets, he wrote: "For this purpose, I took a small dog of three years old, fat, and in good health, and put it to feed upon sugar alone, and gave it distilled water to drink: it had as much as it chose of both . . .

"It appeared to thrive very well in this way of living the first 7 or 8 days; it was brisk, active, ate eagerly, and drank in its usual manner. It began to get meagre upon the second week, though it had always a good appetite, and took about 6 or 8 ounces of sugar in 24 hours . . .

"In the third week its leaness increased, its strength diminished, the animal lost its liveliness, and its appetite was much lessened. At this period there was developed, first upon one eye, and then upon the other, a small ulceration in the centre of the transparent cornea; it increased very quickly, and in a few days it was more than a line in diameter; its depth increased in the same proportion; the cornea was very soon entirely perforated, and the humours of the eye ran out. This singular phenomenon was accompanied with an abundant secretion of the glands of the eyelids.

"It, however, became weaker and weaker, and lost its strength; and though the animal took from 3 to 4 ounces of sugar every day, it became at length so weak that it could neither chew nor swallow; for the same reason every other motion was impossible. It expired the 32nd day of the experiment. I opened it with every suitable precaution; I found a total want of fat; the muscles were reduced by more than five-sixths of their ordinary size; the stomach and the intestines were also much diminished in volume, and strongly contracted . . .

"The excrements, that were also examined by M. Chevreul, contained very little azote, whilst they generally present a great deal . . .

"A third experiment produced similar results, and thence I considered sugar incapable of supporting dogs of itself." Other food substances were also tried (butter gave rise to some ulceration but olive oil did not). Rabbits, guinea pigs, an ass and a cock served as experimental animals to substantiate the findings with dogs. The only noteworthy difference between this and much contemporary research lies in the fact that the amount of work which Magendie crowded into his papers and later into his book would today appear as an avalanche of separate publications.

It has been argued that Magendie might be regarded as the father of vitaminology.[6] Indeed a strong case can be made for the contention that the ulcerations observed in his dogs on protein-free diets were due to nutritional deficiency. However, if one is to be consistent in one's arguments, then Charles Bell had the germ of an idea about spinal root function but failed to recognize its significance, much less fully exploit it; and so did François Magendie have the indications of a nutritional deficiency but failed to recognize its implications. Let it be admitted here that it was not likely that with the scanty information then available about the chemical composition of foods he could have realized the existence of accessory food substances. Indeed, he is not noted for keenness of perception, having had at his disposal all the facts concerning the anesthetic properties of ether without so much as speculating further upon the subject. His genuine contributions afford him sufficient fame to render it unnecessary for us to assign to him the credit of having discovered vitamins.

Other passages from his book are exciting: "Certain substances, but particularly iodine, appear to have a marked influence over nutrition. Their use accelerates or diminishes

[6] McCay, C. M. Science, *71:* 315, 1930.

it. This last effect is very manifest in iodine, and merits special attention."

During Magendie's lifetime Prout was to make history by furnishing the correct classification of foods as saccharine, oleagenous and albuminous. Yet even prior to this, Magendie was to raise the question of substances which furnish no energy but nevertheless contribute to the nutrition of the animal. "Such are the muriate of soda, the oxide of iron, silica, and particularly water."

The use of gelatin as a food had been suggested during the Revolution, the idea having found immediate favor with an economy-minded government. Frequently used in public hospitals in place of meat, it did not appear to meet with the wholehearted acceptance its manufacturers had hoped for. A memoir read before the Academy of Sciences in 1831 reopened the entire question. Some 10 years later the committee submitted its excellent report: gelatin could not replace meat and animals would not voluntarily eat it. Magendie had been a member of the committee.

The up-to-date tone of Magendie's textbook is nowhere better illustrated than in the passage in which he discusses nutrients as being essential to replace substances lost in the ordinary wear-and-tear of metabolism: "On the other side, daily observation teaches, that the organs of man, as well as those of all living beings, lose, at each instant, a certain quantity of that matter which composes them: nay, it is on the necessity of repairing these habitual losses that the want of aliment is founded. From these two data, and some others which we shall make known afterwards, we justly conclude, that living bodies are by no means composed always of the same matter at every period of their existence; physiologists have even gone so far as to say, that bodies undergo an entire renovation."

Other passages, while perhaps not as enlightening scientifically, shed some light on the man's character. "Moral satisfaction, cheerfulness, and mirth, on the contrary, favour digestion: great eaters are seldom accessible to sorrow."

Sorrow seems seldom to have had access to Magendie. The kindliness which he displayed in his private life (but not in his scientific encounters) is to a degree reflected in the frequent passages of the Précis referring to unusual physiological conditions in the very young and the aged. Perhaps one might call Magendie the father of pediatrics and geriatrics.

His outspokenness is well illustrated in an early passage of the Précis: "The useful object of cookery is to render aliments agreeable to the senses, and of easy digestion; but it rarely stops here: frequently with people advanced in civilization its object is to excite delicate palates, or difficult tastes, or to gratify vanity. Then, far from being a useful art, it indeed exerts a great social influence, and contributes somewhat to the comfort and improvement of society; but oftener becomes a real scourge, which occasions a great number of diseases, and has frequently brought premature death."

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COCARBOXYLASE AND THIAMINE IN TISSUES OF RATS RECEIVING DIFFERENT CONCENTRATIONS OF VITAMIN B_1 IN THE DIET

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Several investigators have demonstrated that changes in the dietary concentration of thiamine hydrochloride (vitamin B_1) influence the tissue concentrations of both cocarboxylase and thiamine in several species (Simola, '32; Westenbrink, '32; Ochoa and Peters, '38). However, in none of these studies has the vitamin B_1 concentration in the diet been accurately established, since natural foodstuffs were used and no analyses for vitamin B_1 were made. Thus, no study has been made to compare known dietary intakes of vitamin B_1 with tissue concentrations of thiamine and cocarboxylase.

Thiamine appears to have essentially two physiological functions in tissues. The first, and probably the more important, is its participation in the form of a pyrophosphorylated ester (cocarboxylase) as a coenzyme in several enzyme systems. The second is its function as an activator for these enzyme systems (Ochoa and Peters, '38; Lipschitz et al., '38). It has also been demonstrated by these investigators that enzymes dependent upon cocarboxylase and thiamine are active in direct proportion to the concentration of both components. If the view is taken that the maximum activity of tissue enzyme systems may be an index of vitamin requirements, then a comparison of dietary intake of vitamin B_1 with

[1] The experimental data in this paper were taken from a thesis presented by John H. Flokstra in partial fulfillment of the requirements for the degree of Master of Science.

tissue concentrations of cocarboxylase and thiamine might be used as a method for ascertaining the requirement of a species for the vitamin. Previous studies (Arnold and Elvehjem, '38; Light and Cracas, '38) concerned with the dietary requirement for vitamin B_1 of the rat have measured the intake of the vitamin necessary to support what was considered to be normal growth.

The present study was therefore undertaken to ascertain, first, the effect of the concentration of vitamin B_1 in purified diets upon the tissue concentrations of thiamine and cocarboxylase in rats, and secondly, to compare the dietary requirements of vitamin B_1 for growth with the dietary concentration of thiamine necessary to give a maximum concentration of cocarboxylase and thiamine in rat tissues.

EXPERIMENTAL

Animals and diets

Male and female albino rats of a strain developed in the Michigan State College Chemistry Department, approximately 35 days old, were used as the experimental animals. To avoid the availability of extraneous food material the animals were kept in individual metal cages. For growth experiments the rats were weighed at the beginning and end of the experimental feeding periods.

Purified diets which differed only in their thiamine content were fed these animals for a period of about 4 weeks. All the experimental diets contained the following constituents: casein [2] (vitamin-free) 18%, dextrose 74%, corn oil [3] 4%, and salt mixture (Phillips and Hart, '35) 4%. Vitamins dissolved in 70% ethanol were added by dripping the alcoholic solution from a pipette onto the casein and allowing the casein to dry.

[2] Vitamin-free casein contains a small quantity of thiamine. Analysis of the Labco vitamin-free casein used in these experiments indicated that approximately 20 μg of thiamine per 100 gm of casein were present. This quantity of the vitamin would supply about 0.004 mg of thiamine hydrochloride per 100 gm of the experimental diets used.

[3] Mazola.

The following vitamins were added per 100 gm of all the diets: riboflavin 0.5 mg, pyridoxine 0.2 mg, choline chloride 50.0 mg, calcium pantothenate 2.8 mg.

Four experimental diets were then devised, each containing the above constituents and, in addition, a given amount of thiamine hydrochloride, as shown in table 1. Vitamins A, D, E and K were supplied by feeding each animal two drops (approximately 0.1 ml) of haliver oil per week.

These diets were fed in special cups to prevent spilling, and the approximate weight of food consumed by the experimental animals was measured. Distilled water was supplied for drinking.

TABLE 1

Experimental diets containing different concentrations of thiamine hydrochloride

DIET NUMBER	VITAMIN B_1 (thiamine hydrochloride)
	mg/100 gm of diet
1	0.0
2	0.1
3	0.2
4	0.4

Analytical method for determining cocarboxylase and thiamine

The cocarboyxlase and thiamine concentrations in rat liver, muscle and brain were measured. Rats which had eaten the experimental diets for the designated time period were decapitated and the liver, brain and muscle (biceps femoris) from a hind leg were removed immediately and frozen by being placed on solid carbon dioxide. These three tissues were chosen because in each of them the role of thiamine pyrophosphate as a coenzyme has been demonstrated (Ochoa and Peters, '38; Passmore et al., '33; Abderhalden and Wertheimer, '32). The tissues were kept frozen at $-10°$C. until immediately before they were to be analyzed.

Two weighed tissue samples were homogenized in distilled water (about 1 wt. tissue/3 wt. H_2O), using the Potter-Elvehjem technique (Potter and Elvehjem, '36). Analysis was then made on one whole homogenate for thiamine, using a modification of the method of Hennessey and Cerecedo ('39). The second homogenate was used for an analysis of cocarboxylase, using an adaptation of the same method. This method involved first the fluorometric analysis of thiochrome obtained from oxidation of free tissue thiamine with ferricyanide. The free thiamine concentration was thus obtained directly. Hydrolysis of the pyrophosphate group of cocarboxylase in the presence of phosphatase [4] and oxidation of the thiamine thus obtained plus the thiamine already present as described above, allowed a calculation of cocarboxylase concentration by difference.

EXPERIMENTAL RESULTS

Cocarboxylase analyses

The analyses of muscle, liver and brain of rats receiving diets containing 4 different concentrations of thiamine hydrochloride are presented in column 3 of table 2. The results are expressed as micrograms of cocarboxylase (thiamine pyrophosphate) per gram wet weight of tissue. The tissue concentration was based upon the wet weight since it was necessary to use the whole organ in each case to obtain the desired degree of accuracy in the analytical method. It seemed possible that the results might be expressed more accurately as micrograms of cocarboxylase per gram of tissue nitrogen. Because the whole tissue was needed for cocarboxylase and thiamine analysis, the nitrogen determination (Kjeldahl) had to be made on a tissue sample from a presumably similar animal. An error was thus introduced. Nevertheless, this method of expressing the results was tried but did not, in any way, change the conclusions drawn.

It may be seen from the data that the concentrations of cocarboxylase in all three tissues increased as the concentra-

[4] Clarase.

tion of vitamin B_1 in the diet was raised from 0.0 mg/100 gm to 0.2 mg/100 gm. A further increase in dietary vitamin B_1 to 0.4 mg/100 gm, however, did not increase the concentration of cocarboxylase in these tissues. It was found that within a given group of animals on the same diet no detectable difference in tissue concentrations of cocarboxylase between males and females could be noted.

TABLE 2

Average tissue concentration of cocarboxylase and thiamine

DIET NO.	TISSUE	COCARBOXYLASE	THIAMINE
		μg/gm wet wt.	*μg/gm wet wt.*
1	liver	0.70 ± 0.06 [1]	0.04 ± 0.02 [1]
1	muscle	0.45 ± 0.10 [1]	0.02 ± 0.01
1	brain	1.50 ± 0.13 [1]	0.10 ± 0.06 [1]
2	liver	2.48 ± 0.50 [1]	0.45 ± 0.19
2	muscle	0.32 ± 0.13 [1]	0.22 ± 0.11
2	brain	2.13 ± 0.35 [1]	1.27 ± 0.25
3	liver	6.27 ± 0.56	0.88 ± 0.21
3	muscle	1.43 ± 0.24	0.07 ± 0.09
3	brain	4.23 ± 0.62	1.09 ± 0.37
4	liver	6.22 ± 0.87	2.94 ± 0.84 [1]
4	muscle	1.31 ± 0.42	0.94 ± 0.37 [1]
4	brain	3.38 ± 0.53	1.19 ± 0.41

[1] Indicates a significant difference from the values listed for diet 3 by "t" test for significance. The ± values are one standard error of a mean of 6 analyses in each case.

A statistical analysis was made of the data to observe any statistically significant differences between tissue cocarboxylase concentrations. Since maximum tissue concentrations of cocarboxylase resulted when 0.2 mg vitamin B_1 per 100 gm of diet were fed, it appeared feasible to compare these values with the tissue concentration obtained when the other three diets were fed. The tissue cocarboxylase concentrations found when diets 1 and 2 were fed were all significantly lower than the concentrations found after diet 3 was fed, as judged by the "t" test for significance. However, feeding diet 4 gave no

significant difference in cocarboxylase concentration from that obtained with diet 3.

It will be noted that the analyses for cocarboxylase were made in tissues which had been frozen for a period of one to two months. To ascertain the extent of destruction of cocarboxylase which might have occurred while the tissues were in a frozen state, analyses were made on a portion of an organ just after the animal was sacrificed, and again a month later upon a remaining portion which had been frozen. The results of such analyses indicated that no measurable destruction of cocarboxylase had occurred in the frozen samples.

Thiamine analyses

An analysis for thiamine was also made upon muscle, liver and brain of rats receiving experimental diets containing 4 different concentrations of vitamin B_1. These data are presented in column 5 of table 2. The results are expressed as micrograms of thiamine (free thiamine) per gram of wet tissue weight (see above). In all these tissues the concentration of thiamine was less than the cocarboxylase concentration. In liver there was a progressive increase in the concentration of thiamine as the dietary vitamin B_1 concentration increased up to 0.4 mg/100 gm. The same trend was noted in muscle tissue. By comparison, in the brain there was no increase in thiamine when the concentration of vitamin B_1 in the diet exceeded 0.1 mg/100 gm.

Again a statistical analysis was made of the data to ascertain any significant statistical differences in tissue concentrations of thiamine. Since diet 3 had been used for comparison in analyzing the cocarboxylase concentration, it was also used with thiamine. It will be noted that only the concentrations of thiamine in the liver and brain of animals receiving no added vitamin B_1 were significantly lower than the concentration after diet 3 had been fed, even though there appear to be small differences when the other two diets were fed. When diet 4 was fed, both liver and muscle contained significantly higher concentrations of thiamine.

Growth studies

Weight gains during the feeding period were measured in order to ascertain how the vitamin B_1 requirement for the growth of rats compared with the dietary concentration of vitamin B_1 necessary to give maximum tissue concentrations of cocarboxylase and thiamine. The growth data for rats receiving the 4 experimental diets for 28 days are presented in

TABLE 3

Average weight gain and daily food intake of rats receiving experimental diets

DIET NO.	NUMBER AND SEX	AVERAGE INITIAL WEIGHT	AVERAGE WEIGHT GAINED	AVERAGE DAILY FOOD INTAKE
		gm	*gm/28 days*	*gm/day*
1	3 ♂ 3 ♀	68 ± 2	— 1.9 ± 1.9[1]	6.2 ± 0.3[1]
2	4 ♂ 2 ♀	65 ± 3	96 ± 11	10.7 ± 0.4
3	4 ♂ 2 ♀	57 ± 2	115 ± 6	11.6 ± 0.3
4	4 ♂ 2 ♀	64 ± 2	102 ± 8	10.6 ± 0.4

[1] Indicates a significant difference from the values listed for diet 3 by "t" test for significance. The ± values are one standard error of the mean of 6 determinations in each case.

table 3. It will be noted that there was no significant difference in the growth of animals receiving diets 2, 3 and 4. It may therefore be concluded that normal growth resulted when about 0.1 mg vitamin B_1 per 100 gm of diet was fed. These data are essentially in agreement with the findings of Arnold and Elvehjem ('38), who established the minimum requirement of vitamin B_1 for the normal growth of the rat to be 0.08 to 0.10 mg thiamine hydrochloride per 100 gm of diet.

DISCUSSION

It is apparent from the data presented that as the concentration of vitamin B_1 in the diet was increased the concentra-

tions of both cocarboxylase and thiamine in rat liver, brain and muscle also increased. However, the tissue concentrations of cocarboxylase reached a maximum when 0.2 mg vitamin B_1/100 gm of diet were fed. In contrast, maximum tissue thiamine concentrations were not reached over the range of intakes of vitamin B_1 employed, except in brain tissue. When no thiamine was added to the basal ingredients of the diet, some cocarboxylase and thiamine were noted in the rat tissues. It has been pointed out that the diet to which no vitamin B_1 was added contained a small quantity of thiamine hydrochloride, which would contribute in a limited way to the supply of cocarboxylase and thiamine. It is possible also that some bacterial synthesis of thiamine occurred in the rat intestine, although the use of glucose as the only dietary carbohydrate would minimize this source of the vitamin. In addition, limited biochemical synthesis, retention or redistribution of thiamine by the rat itself cannot be ruled out. The animals receiving no vitamin B_1 supplement, however, showed severe symptoms of vitamin B_1 deficiency.

In measuring the vitamin B_1 requirement by the growth method, it was noted that about 0.1 mg vitamin B_1 per 100 gm of diet gave normal growth. Feeding diets containing 0.2 and 0.4 mg/100 gm resulted in no statistically significant differences in growth. These results are essentially in accord with those of Arnold and Elvehjem ('38), who indicated that the minimum vitamin B_1 requirement for rat growth was 0.08 to 0.10 mg/100 gm of diet. In contrast to the results with growth, 0.2 mg vitamin B_1/100 gm of diet were necessary to give maximum cocarboxylase and thiamine concentrations in the tissues, whereas 0.1 mg vitamin B_1 per 100 gm of diet gave significantly less tissue cocarboxylase as well as less thiamine. It would appear, then, that the dietary concentration of vitamin B_1 required for normal growth does not provide adequate material for sustaining maximum cocarboxylase or thiamine concentrations in the tissues. It has been adequately demonstrated (Ochoa and Peters, '38; Lipschitz et al., '38) that enzymes dependent upon either cocarboxylase or

thiamine are active in direct proportion to the presence of both of these components. It is apparent that dietary intakes of vitamin B_1 in excess of the amount necessary to support normal growth are essential to attain maximum enzyme activity. It is possible that the dietary quantities of vitamin B_1 which give optimum enzyme activity may be necessary for adequate metabolism under certain conditions.

SUMMARY

The influence of the concentration of vitamin B_1 in purified diets upon rat tissue concentrations of cocarboxylase and thiamine has been demonstrated. As the dietary concentration of vitamin B_1 was increased up to 0.2 mg per 100 gm of diet, the concentration of cocarboxylase also increased in brain, muscle and liver. However, a dietary concentration of vitamin B_1 greater than 0.2 mg per 100 gm of diet gave no further increase in tissue cocarboxylase. In contrast, as the dietary concentration of vitamin B_1 was increased to 0.4 mg per 100 gm of diet, the tissue concentration of thiamine also increased, except in the brain.

The vitamin B_1 requirement for rat growth was compared with the dietary vitamin B_1 concentration necessary to give maximum tissue concentrations of cocarboxylase and thiamine. In agreement with other workers, about 0.1 mg of vitamin B_1 per 100 gm of diet was required to support normal growth. However, at least 0.2 mg of vitamin B_1 per 100 gm of diet were required to give maximum cocarboxylase levels, and tissue thiamine concentrations increased up to 0.4 mg of vitamin B_1 per 100 gm of diet, except in brain tissue.

ACKNOWLEDGMENT

The authors wish to thank Dr. K. B. McCall of this Department for his helpful criticism and advice.

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SOME NUTRITIONAL REQUIREMENTS OF ADULT MOSQUITOES (AEDES AEGYPTI) FOR OVIPOSITION

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TWO FIGURES

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Although Fielding ('19) obtained eggs from *Aedes aegypti* fed on a peptone-sugar water solution, neither Young ('22) nor Gordon ('22) was able to repeat his results. A search of the literature reveals no other report of this species being able to oviposit without a blood meal.

Woke ('37) obtained eggs from *A. aegypti* females fed either the corpuscular or serous fraction of rabbit blood. It was felt that information as to the nutritional requirements of the mosquito could be obtained by continuing this line of investigation.

MATERIALS AND METHODS

The *Aedes aegypti* used in these experiments were reared according to the methods described by Trembley ('44). To the larval rearing pans were added ground dog biscuits, beef brain-heart infusion [1] and brewers' yeast [2]. Four to 5 days after emergence the adult females were lightly anesthetized and groups of 20 or 30 were placed in lantern globe cages. The mosquitoes were allowed to recover over night with no access to food or water. The following day they were allowed to feed on the test media.

[1] Difco.

[2] Harris.

A method for artificially feeding mosquitoes has already been described in some detail (Greenberg, '49) and will be mentioned only briefly here. Small feeding vials containing the test fluids were warmed to about 40°C. by electrically heated resistance wire coils. Each feeding vial was open at one end and covered at the other end with dehydrated chick skin (Bishop and Gilchrist, '44, '46) or Baudruche Capping Membrane. The feeding vials were placed, membrane side down, on top of the lantern globe cages. Each vial was surrounded by a warming coil. The mosquitoes were allowed access to the food for one-half hour, after which time the numbers which had fed were noted and the cages placed in an incubator at 30°C. and 70% relative humidity. Wet filter paper was provided for oviposition, which generally occurred 5 to 6 days after feeding. During this time the mosquitoes subsisted on 5% glucose. The number of fed mosquitoes that died before the egg laying period was over was subtracted from the original number which had fed. In no instance was mortality significant. On the 7th day after feeding the eggs were collected and counted. An estimate of the number of eggs per female was made by dividing the total number of eggs laid by the number of mosquitoes that had fed. Every diet was tested at least twice, on different days. For each series of diets tested on one day, one group of mosquitoes received defibrinated sheep blood or suspended sheep erythrocytes. The data have been grouped into related units. Occasionally batches of eggs were tested for viability, and in all instances more than 75% hatched.

EXPERIMENTAL

Mosquitoes did not feed on the serum or plasma of rabbits, chickens or man, in repeated trials. However, they ingested erythrocytes suspended in saline as readily as defibrinated blood. The oviposition resulting from feeding defibrinated blood and washed erythrocytes of chicks, rabbits, sheep and man is shown in table 1. It will be noted that mosquitoes fed

TABLE 1

The effect of defibrinated blood, and physiological saline suspensions of erythrocytes, of various animals on oviposition of Aedes aegypti

MEDIUM	NO. OF TRIALS	NO. OF MOSQUITOES FED	EGGS/FEMALE (mean)
Rabbit blood	4	46	65.6 ± 13 [2]
Chick blood	5	40	95.8 ± 5.5
Sheep blood	38	362	81.5 ± 2.2
Human blood	9	101	47.5 ± 3.1
Rabbit erythrocytes [1]	12	172	47.3 ± 1.0
Chick erythrocytes	4	87	84.7 ± 8.4
Sheep erythrocytes	19	315	4.6 ± 1.0
Human erythrocytes	4	104	27.1 ± 18

[1] The erythrocytes of each of these animals were washed three times in 0.9% NaCl and resuspended in physiological saline to the original volume of blood.

[2] Standard error of the means.

TABLE 2

The effect of washed sheep erythrocytes, variously supplemented, on oviposition of Aedes aegypti

(The supplementary media were added to thrice-washed sheep erythrocytes up to the original volume of sheep blood)

SUPPLEMENTARY MEDIUM [1]	NO. OF TRIALS	NO. OF MOSQUITOES FED	EGGS/FEMALE (mean)
A	3	63	4.4 ± 3.03 [2]
B	2	34	4.6 ± 3.30
C	3	52	3.6 ± 0.90
D	2	34	6.7 ± 2.23
E	2	52	7.0 ± 1.64
F	3	51	19.0 ± 3.30
G	3	49	12.1 ± 1.20

[1] *Medium A* (Ringer's solution): NaCl, 8.0 gm; KCl, 0.2 gm; $CaCl_2$, 0.2 gm. Distilled H_2O up to 1,000 ml. *Medium B* (Locke's solution): NaCl, 8.0 gm; KCl, 0.2 gm; $CaCl_2$, 0.2 gm; $MgCl_2$, 0.01 gm; Na_2HPO_4, 2.00 gm; $NaHCO_3$, 0.40 gm; KH_2PO_4, 0.3 gm; distilled H_2O to 1,000 ml. *Medium C*: to 100 ml of medium B add Mn (as $MnSO_4$) 100 μg; Cu (as $CuSO_4$) 150 μg; Zn (as $ZnSO_4$) 10 μg; I (as NaI) 10 μg. *Medium D*: medium C plus glucose, 100 mg per 100 ml. *Medium E*: to 100 ml of medium D add thiamine HCl, 20 μg; nicotinic acid, 800 μg; riboflavin, 50 μg; pyridoxine HCl, 20 μg; *p*-aminobenzoic acid, 20 μg; biotin ethyl ester, 5 μg; pteroylglutamic acid, 5 μg; choline chloride, 2 mg; *i*-inositol, 1 mg. *Medium F*: to 100 ml of medium E add crystalline bovine plasma albumin (Armour) 100 mg. *Medium G*: to 100 ml of medium F add 0.5 ml Natola (Parke-Davis) containing 25,000 U.S.P. units of vitamin A and 5,000 U.S.P. units of vitamin D.

[2] Standard error of the means.

suspended sheep erythrocytes laid only about one-sixteenth as many eggs as those fed defibrinated sheep blood.

In an attempt to define the essential food requirements for oviposition, various substances were added consecutively and cumulatively to washed sheep erythrocytes. Table 2 shows that

TABLE 3

Effect of sheep erythrocytes supplemented with various proteins and protein products on oviposition of Aedes aegypti

(The supplementary media were added to thrice-washed sheep erythrocytes made up to the original volume of sheep blood)

SUPPLEMENT USED WITH SHEEP ERYTHROCYTES	NO. OF TRIALS	NO. OF FEMALES FED	EGGS/FEMALE (mean)
Albumin, bovine plasma, 5% [1]	15	73	52.4 ± 3.13 [5]
Albumin, human plasma, 5% [2]	5	83	42.7 ± 1.25
Albumin, egg 5% [3]	4	29	61.0 ± 0.83
Globulin, human gamma, 5% [4]	10	151	48.0 ± 2.49
Gelatin, 5% [5]	4	46	42.5 ± 3.07
Hemoglobin, crystalline horse, 5%	2	30	8.4 ± 0.60
Casein, 2% [6]	8	62	37.8 ± 1.97
Casein, tryptic digest, 2%	7	62	30.7 ± 2.15
Bacto-peptone, 5% [7]	2	16	15.6 ± 2.00

[1] Armour Laboratories, Armour and Company. Lot 46.

[2] Diluted from 25% albumin solution in buffered diluent with 1: 10,000 merthiolate. Lot 33019D, Eli Lilly Company.

[3] Merck, impalpable powder, soluble.

[4] Diluted from solution containing 0.29 gm protein/ml. Normal serum gamma globulin antibodies (human). Prepared by the Department of Physical Chemistry, Harvard Medical School. Fraction II in physiological saline. Merthiolate 1: 10,000. Preparation 11GS74B2, June 8, 1945.

[5] J. T. Baker Chemical Company. U.S.P. powder, lot 82644.

[6] Labco, vitamin-free.

[7] Difco.

[8] Standard error of the means.

no significant increase in oviposition occurred until a small amount of bovine plasma albumin was added to the medium. Oviposition increased in proportion to the concentration of bovine plasma albumin up to 5 gm/100 ml (fig. 1). Other proteins derived from blood or other sources, and certain protein products, were able, in varying degrees, to increase oviposition

over that which followed ingestion of washed sheep erythrocytes alone (table 3). The small effect obtained from the addition of horse hemoglobin should be noted.

Apparently a significant portion of the oviposition-stimulating substance which occurred in sheep plasma could be supplied by any one of a number of protein preparations. A series of experiments was undertaken to determine whether

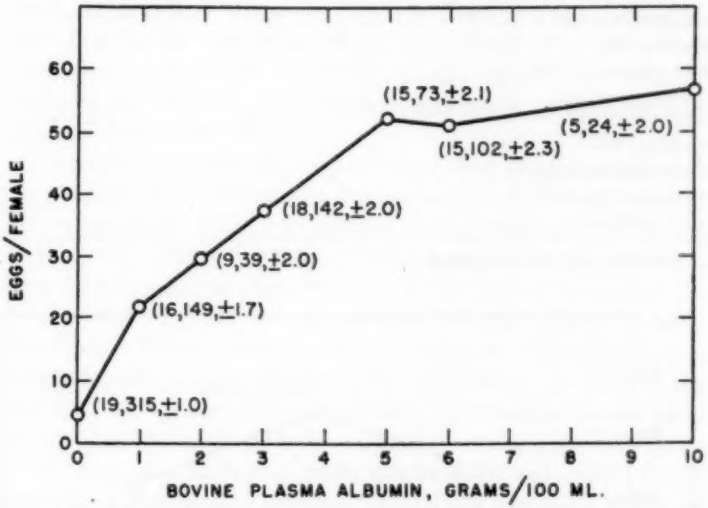

Fig. 1 The effect of washed sheep erythrocytes, supplemented with various concentrations of bovine plasma albumin, on oviposition by *Aedes aegypti*. The figures beside each point give the number of trials, number of mosquitoes fed, and standard error of the means.

the protein could be replaced by a mixture of amino acids. All the details of these experiments can hardly be presented, but it can be said that a significant increase in oviposition followed the addition of DL-isoleucine to sheep erythrocytes, but no increase followed the addition of a mixture of 9 other acids (table 4). The number of eggs per female increased wth increasing supplements of DL-isoleucine up to 1 gm per 100 ml (fig. 2). It will be noted, however, that even large amounts of

TABLE 4

The effect of washed sheep erythrocytes supplemented with amino acids on oviposition of Aedes aegypti

(The amino acid supplements in 0.85% NaCl were added to thrice-washed sheep erythrocytes to equal the original volume of sheep blood)

AMINO ACIDS	NO. OF TRIALS	NO. OF FEMALES FED	EGGS/FEMALE (mean)
mg/100 ml			
DL-isoleucine, 300	30	438	24.2 ± 0.80 [1]
DL-methionine, 200 DL-valine, 600 DL-phenylalanine, 500 DL-tryptophan, 200 DL-threonine, 600 DL-leucine, 900 L(+) arginine, 600 L(—) histidine, 250 L(+) lysine, 400	4	19	4.2 ± 3.0

[1] Standard error of the means.

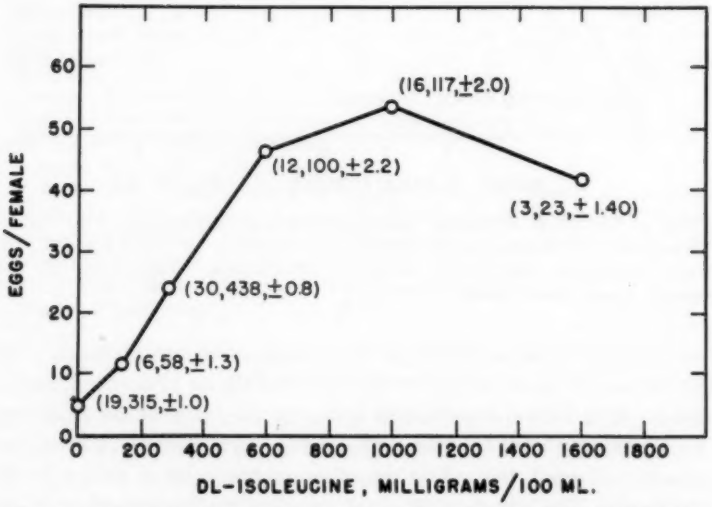

Fig. 2 The effect of washed sheep erythrocytes, supplemented with DL-isoleucine, on oviposition by *Aedes aegypti*. The figures beside each point give the number of trials, number of mosquitoes fed, and standard error of the means.

isoleucine failed to increase oviposition to the level obtained by feeding defibrinated sheep blood (table 1).

DISCUSSION

The problem of the nutritional requirements for oviposition of the adult female *Aedes aegypti* has been little investigated since the work of Woke ('37). It is a subject with many difficulties, chief of which is that it is practically impossible by our methods to induce the mosquitoes to feed on fluids which contain few or no whole erythrocytes. Until this difficulty is overcome, completely synthetic media cannot be fed. The problem can, however, be examined indirectly by such means as are reported in this paper.

Preliminary trials showed that washed sheep erythrocytes were markedly deficient in some factor or factors required for oviposition. Of the inorganic salts, vitamins, glucose, fat and protein added to sheep erythrocytes, only the protein increased oviposition significantly. Oviposition increased proportionally with the concentration of bovine plasma albumin up to 5%. Of the several proteins tested at similar concentrations, egg albumin and human gamma globulin were at least as effective as bovine plasma albumin. Crystalline horse hemoglobin was the least effective, scarcely increasing oviposition at all over that obtained with unsupplemented cells. In this connection the results of Yoeli and Mer ('38) may be significant. They found that *Anopheles elutus* failed to oviposit when fed washed donkey erythrocytes. It is possible that equine hemoglobin is as nutritionally deficient as are sheep erythrocytes. Whole protein could to a large extent be replaced by DL-isoleucine, but not by a mixture of some 9 other amino acids considered to be essential in the nutrition of higher animals (Rose, '38). This would indicate that sheep serum furnishes isoleucine to erythrocyte proteins deficient in this amino acid. There is no information available on the amino acid content of sheep erythrocytes, or on the nutritional value of the sheep red cell fraction for higher animals. However, it has been found that human hemoglobin is a deficient protein for regen-

eration of protein in dogs (Whipple, '42) and will not support growth in rats (Devlin and Zittle, '44). Albanese ('45) found that human hemoglobin supplemented with isoleucine would allow growth in rats.

It is obvious that the limits of the present method of investigation have not been reached. Assuming that oviposition following the ingestion of chick blood represents a maximum achievement by the mosquito, only 50% of this maximum resulted when the mosquitoes were fed sheep erythrocytes supplemented with the experimentally determined optimum concentration of isoleucine. This concentration of isoleucine is probably much larger than occurs normally in sheep plasma. Some of the differences observed between maximum oviposition and that achieved with the best of the semi-artificial media may be attributable to reduced food intake. This factor will have to be examined. Furthermore, there are vitamins, amino acids and other food substances (purines and pyrimidines, for example) which were not tested, but which would bear investigation.

One other observation was made during the course of these experiments which was not followed up but which may prove of value in determining the oviposition factors contained in the erythrocyte. It was found that *Aedes aegypti* would readily ingest a saline solution containing very small numbers (one-sixteenth to one-thirty-second original volume) of erythrocytes. By supplementing such diluted suspensions of erythrocytes it may be possible to determine other nutritional requirements of the adult female mosquito.

SUMMARY

Aedes aegypti laid 16 times as many eggs when fed defibrinated sheep blood as when fed washed sheep erythrocytes. A series of inorganic salts, a series of vitamins, and glucose, when added to sheep erythrocytes, failed to increase oviposition significantly. Several proteins and protein products were able markedly to increase oviposition in mosquitoes fed sheep erythrocytes. The proteins could be replaced almost

completely by isoleucine but not by a combination of some 9 other amino acids. However, even when sheep erythrocytes were supplemented with the optimum concentration of proteins or isoleucine, oviposition did not equal that achieved with defibrinated sheep blood.

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CHOLINE DEFICIENCY IN THE CALF

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FOUR FIGURES

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Choline has been shown to be a dietary essential for the rat (Best and Huntsman, '32) and for the dog (Best and Hershey, '32; Schaefer, McKibbin and Elvehjem, '41) and to be replaceable by a labile methyl source (Du Vigneaud et al., '39).

We have developed in this laboratory a "synthetic milk" ration which is satisfactory for the normal growth of day-old calves (Wiese et al., '47) and of the day-old baby pig (Johnson et al., '48b). Using this "synthetic milk" ration with baby pigs, we have demonstrated the choline requirement of the baby pig (Johnson and James, '48a; Neumann et al., '49) and the replaceability of choline by methionine (Nesheim et al., '49, '50).

In the present paper we are reporting the production of choline deficiency in the calf. Waugh, Hauge and King ('46, '47a, b) have reported that colostrum is relatively high in choline (0.69 mg/ml compared to 0.14 mg/ml for milk) and that while the calf's choline blood level is low at birth, it increases with the ingestion of colostrum. They state that the comparatively high choline content of the colostrum probably aids in the nutrition of the newborn calf.

EXPERIMENTAL AND RESULTS

Ten one- to two-day-old male dairy calves were fed the "synthetic milk" ration given in table 1. Three calves (two Holstein

and one Guernsey) received this diet supplemented with 200 mg of choline chloride per 100 gm dry matter of the diet (260 mg per liter of milk). The other 7 calves (one Ayrshire, one Jersey and 5 Holsteins) received the basal unsupplemented diet.

TABLE 1

Composition of "synthetic milk" ration

BASAL MIXTURE		VITAMIN SUPPLEMENTS [1] ADDED PER LITER OF THE HOMOGENIZED MILK WITH 13% SOLIDS			
	%		mg		
Casein (Labco)	15.0	Thiamine	0.65	*p*-Aminobenzoic acid	2.6 mg
Cerelose	52.4	Riboflavin	1.30	Biotin	0.01 mg
Lard	26.6	Pyridoxine	1.30	α-Tocopherol acetate	1.0 mg
Minerals [2] These ingredients were mixed and homogenized into a milk containing 13% solids.	6.0	Calcium pantothenate	7.8	2-Methyl-1, 4-naphthoquinone	0.26 mg
		Inositol	26.0	Vitamin A	2,000 I.U.
		Nicotinic acid	2.6	Vitamin D_3	200 I.U.
		Folic acid	0.052		

[1] Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid and α-tocopherol acetate were supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cynamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes. Hyflavin (a highly water-soluble form of riboflavin) was supplied by Endo Products, Inc., Richmond Hill, New York.

[2] See Johnson et al., '48b.

The protein level used in this study was lower (15%) than in our previous "synthetic milk" studies (cf. Wiese et al., '47), as we wished to avoid feeding enough methionine to supply all the required labile methyl groups. Three of the calves (65H and 67H on the choline-free diet and 66H on the diet containing choline) received 0.3% cystine in addition to the 15% casein.

However, they presented the same picture as those not receiving the cystine and so will not be considered separately.

In figure 1 are plotted the growth curves of the three calves on the choline-supplemented ration. As can be seen from this chart, while these calves gained rather poorly during their first three or 4 weeks, they did live and grow reasonably well (ave. 0.35 lb. per day gain) on this 15% casein diet.

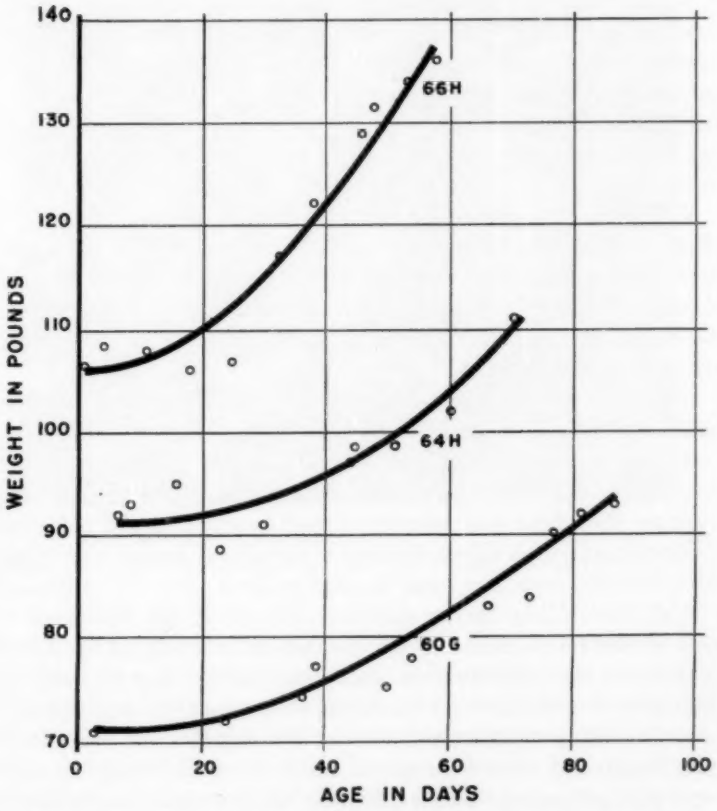

Fig. 1 Growth curves of calves receiving choline in conjunction with a 15% casein ration.

The results observed with the 7 calves fed the choline-free diet are given below as case records of the individual calves.

Calf 55A. This Ayrshire calf was placed on the choline-free diet at two days of age, at which time he weighed 102 lb. The calf ate normally, but after 6 days on the diet he showed weakness and lay down all the time. By the 7th day the calf was very weak and unable to stand, and refused food.

One gram of choline chloride was injected intravenously. The calf immediately fell down in the cage, and showed excessive salivation, labored breathing, and so forth (an "acetyl choline type" reaction). This phase, however, lasted only 10 or 15 minutes, and by the next day, i.e., the 8th day on experiment, the calf appeared much improved and was able to get up by himself. By the 9th day the calf appeared completely recovered.

Calf 57H. This Holstein calf was placed on the choline-free diet at 6 days of age, at which time he weighed 108 lb. He began by eating normally, but after 8 days on the diet was weak and unable to stand. One gram of choline was given by subcutaneous injection, and the calf was placed on the choline-containing diet for 6 days.

The calf showed a gradual recovery and 12 days later was standing by himself and consuming food willingly. From the 38th day on experiment to the 72nd the calf gained 17 lb., an average of 0.5 lb. per day, which is good response considering that on slaughter and autopsy it was found that the calf had pneumonia, which accounted for a persistent cough and probably for the slow response to choline.

Calf 58J. This Jersey calf was placed on the choline-free diet at two days of age, at which time he weighed 50 lb. After 5 days on this diet he was weak and unable to get up alone. However, he could stand when placed on his feet, and therapy was not begun at this time. On the 7th day the calf was found moribund and unable to move, although still breathing. He was given 1 gm of choline (200 mg intravenously and 800 mg subcutaneously), and at 4 p.m. was still breathing but his extremities were cold and his tongue was protruding and cy-

anotic. He was given glucose plus a stimulant intravenously with no effect. Pathological examination showed hemorrhages around the kidneys and the presence of some fat in the liver (see fig. 2). He apparently died of an acute choline deficiency.

Calf 59H. This Holstein calf was placed on the choline-free diet at two days of age, at which time he weighed 100 lb. After 4 days on test this calf appeared weak and had to be assisted

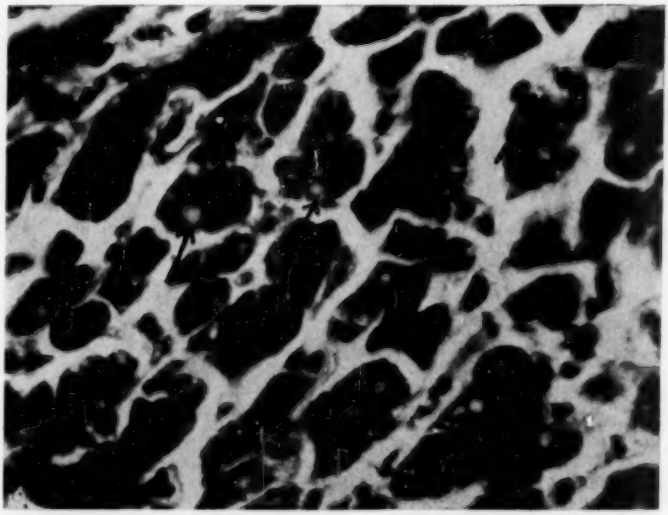

Fig. 2 Calf 58J. Mild fatty infiltration of the liver. The vacuoles in the hepatic cells represent lipoidal material which stained with fat-soluble dyes. Hematoxylin and eosin. × 600.

to stand. By the 5th day he was weaker, had anorexia, an irregular heart rate and respiration, and showed poor muscular coordination, his body swaying unsteadily. By the 6th day the heart rate and respiration were rapid and the calf showed tremors, particularly of the forelegs. On the 7th day 1 gm of choline was injected subcutaneously; however, respiration and heart action were still rapid after several hours and the calf lay with the mouth open and the tongue protruding and

cyanotic and the eyes sunken. At this stage he was given ½ gm choline intravenously. He developed Cheyne-Stokes respiration and died within three minutes.

Pathological examination of this calf showed mild fatty infiltration of the liver (see fig. 3), and the kidney sections revealed evidence of some degeneration of the tubules.

Calf 62H. This Holstein calf was placed on the choline-free diet at two days of age, at which time he weighed 82 lb. After

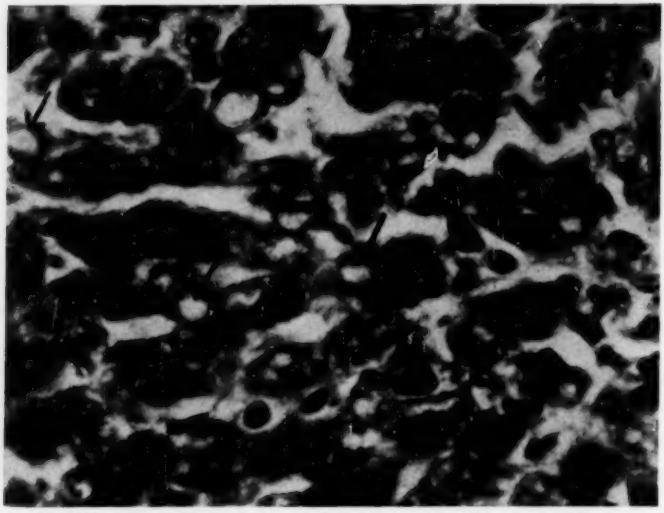

Fig. 3 Calf 59H. Mild fatty infiltration of the liver. Hematoxylin and eosin. × 600.

6 days on the diet this calf was unable to get up by himself, appeared weak and refused food. He was given 1 gm of choline by subcutaneous injection and 5 gm of choline orally. The next day he appeared improved, was able to remain standing after being helped up and consumed food willingly. Two days after the choline therapy he was able to get up by himself. A month later he appeared in good health and spirits, and he was taken off experiment at 108 days. This calf received choline in his

diet for 7 days following the injection and from then on received no further choline; yet he showed no further evidence of a choline deficiency. In this respect he performed similarly to calf 57H.

Calf 65H. This Holstein calf was placed on the choline-free diet at one day of age, at which time he weighed 87.5 lb. Having been taken off the dam at one day of age, he received less

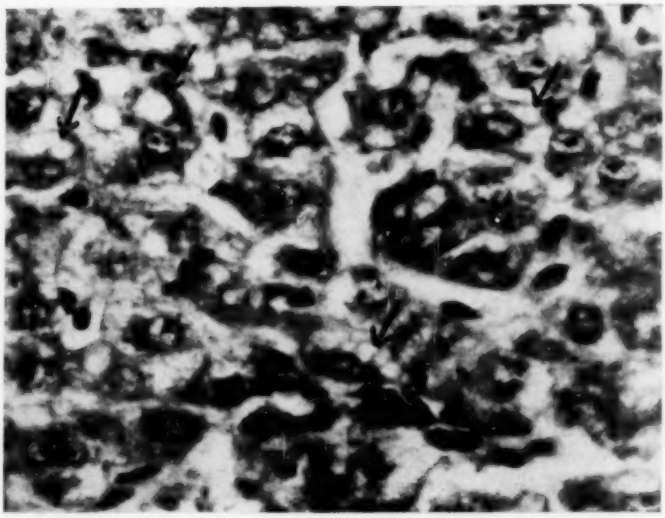

Fig. 4 Calf 67H. Albuminous degeneration and mild fatty changes in the liver. Hematoxylin and eosin. × 600.

colostrum than did the previous experimental animals. After 4 days on the diet the animal had developed anorexia and appeared weak. At 5 days the calf was very weak, was unable to get up, and lay on his side with his mouth open, tongue out and respiration rapid. He was given choline (1 gm intravenously in 10 ml of water and 5 gm orally). The animal did not make a very good recovery, probably because the deficiency was allowed to become too severe before treatment. The next day he was still extremely weak. By the 7th day after therapy

TABLE 2

Average blood data

CALF NO.	HEMOGLOBIN	RED BLOOD CELLS	WHITE BLOOD CELLS	DIFFERENTIAL OF WHITE CELLS				NUMBER OF DETERMINATIONS
				Nucleocytes	Lymphocytes	Mononucleocytes	Eosinophils	
			Positive control calves receiving choline					
	%							
60G	9.71	8,706,000	5,850	23.5	69	6	1.5	12
64H	8.29	7,570,000	7,930	30.3	68.2	1.5	0	7
66H	12.0	9,810,000	8,920	35.5	60.3	3.3	0.5	6
			Calves on basal diet before and after first choline administration [1]					
58J	5.3	4,540,000	7,000	50	47	3	0	1
	8.0			65	35	4	0	1
59H	10.0	10,160,000	14,875	51	45	5	0	2
	10.9	10,925,000	40,800	..	..	..	..	2
62H	8.0	6,900,000	9,000	64	3.3	3	0	2
	7.93	7,860,000	10,300	40.7	55.6	3.6	1	7
65H	9.5	9,300,000	6,000	20	77	3	0	1
	9.6	8,720,000	5,500	64	31	1	0	1
67H	7.0	5,460,000	11,500	46	53	1	0	1

[1] Figures on first line — before therapy; figures on second line — after therapy.

was begun he appeared considerably better but died later that day of severe bloat. Bloat had occurred several times during that week.

Calf 67H. This Holstein calf was placed on the choline-free diet the day he was born and did not receive any colostrum. He weighed 76 lb. He became weak after 4 days on the deficient diet, and after 7 days on the diet was so weak he was unable to get up. At this stage he was removed for autopsy and pathological examination. The liver on histological examination showed rather marked swelling, due partly to albuminous de-

TABLE 3

Urinary choline excretion

CALF NO.	DAYS ON EXPERIMENT	URINARY CHOLINE
		μg/day
	Calves receiving choline	
60G	34	58
66H		94
	17	271
	29	310
	37	484
	47	324
	Calves on choline-free diet	
55A	1	17.4
59H	2	12
62H	6	10.9

generation (cloudy swelling) and in lesser degree to fatty changes (see fig. 4). The kidneys showed diffuse albuminous degeneration.

Blood samples were taken from all calves at approximately weekly intervals. The averages of the blood data obtained are given in table 2. These data show no statistically significant effects of the choline deficiency. However, two of the calves on the choline-free diet (58J and 67H) did have low hemoglobin values, and three calves (58J, 62H and 67H) had low red cell counts. With one exception (one determination on calf 65H), the calves fed the choline-free diet showed a higher percentage

of lymphocytes than did the calves on the choline-containing diet.

Twenty-four-hour urine samples were collected at intervals from many of the calves and urinary choline determinations were made by the *Neurospora crassa* cholineless method of Horowitz and Beadle ('43). The urinary choline excretion data are summarized in table 3. This table shows a low level of choline excretion in those calves not receiving choline in their diets, even though they had recently received colostrum.

DISCUSSION

From the results presented it appears evident that the young calf when fed a 15% casein diet requires a supplementary source of choline. It was surprising to us that the deficiency developed in such a short period of time (6 to 8 days) and was so acute. This happened even though the calves had received colostrum (from one to 6 days with the cow), which Waugh et al. ('46, '47a) have shown to be a good dietary choline source. In the two calves (57H and 62H) which were kept on experiment for several weeks, body choline synthesis appeared adequate once the initial critical period was past.

Symptoms of renal hemmorrhage were observed twice (calves 58J and 67H). Slightly fatty livers were observed in three cases (calves 58J, 59H, and 67H). Perhaps a diet slightly higher in protein and slower development of the deficicency would have resulted in greater severity of this syndrome. However, Handler ('49) did not observe fatty livers in choline-deficient guinea pigs, and the guinea pig is probably physiologically closer to the calf than is the rat. Also, the three calves, 55A, 57H and 62H, which were kept on experiment for 16, 47 and 51 days, respectively, after choline had been removed from their diets (following the original therapy) showed no fatty infiltration of the liver.

SUMMARY

On a "synthetic milk" diet containing 15% casein or 15% casein plus 0.3% cystine as the nitrogen source, two-day-old

dairy calves were found to require a supplementary source of choline. When choline was omitted from the diet, an acute deficency syndrome developed in about 7 days. The symptoms included marked weakness and inability to get up, labored or rapid breathing and anorexia. Unless the deficiency had progressed too far, the calves responded to choline therapy. Later removal of choline from the diet did not appear to have any harmful effects.

ACKNOWLEDGMENTS

We are grateful to Dr. L. E. Boley of the College of Veterinary Medicine for his help in examining the calves clinically and to Miss Emily L. Dorstewitz for histopathological processing of tissues.

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STUDIES ON THE VITAMIN B_{12} CONTENT OF FEEDSTUFFS AND OTHER MATERIALS [1, 2]

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THREE FIGURES

(Received for publication June 3, 1950)

Lillie, Denton and Bird ('49a), Lillie, Denton, Olsen and Bird ('49b) and Ott ('49) have studied the vitamin B_{12} potency of feedstuffs and concentrates by chick growth assay. The vitamin B_{12} content of natural materials has also been studied by Hartman, Dryden and Cary ('49), Lewis, Register and Elvehjem ('49a) and Lewis, Register, Thompson and Elvehjem ('49b) by rat assay. Ansbacher, Hill, Tieman, Downing and Caldwell ('49) have made determinations of the vitamin B_{12} content of fish meals and fish solubles, using a microbiological assay. More recently, Peeler, Yacowitz and Norris ('49) have ascertained the vitamin B_{12} content of a diversity of materials by use of an improved microbiological assay. However, Stokstad et al. ('49) were unable to obtain

[1] A preliminary report of this study was presented at the 117th meeting of the American Chemical Society, Philadelphia, Pennsylvania, April 9-13, 1950.

[2] This work was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, the Cerophyl Laboratories, Inc., Kansas City, Mo., the Commercial Solvents Corporation, New York City, the Cooperative G. L. F. Exchange, Ithaca, N. Y., and the Western Condensing Company, San Francisco, California. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

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satisfactory correlation between the values obtained microbiologically and those obtained by animal bioassay.

Thymidine and other desoxyribosides have been reported (Shive, Ravel and Eakin, '48; Snell, Kitay and McNutt, '48; Wright, Skeggs and Huff, '48; Shive, Ravel and Harding, '48; and Kitay, McNutt and Snell, '49) to possess vitamin B_{12} activity for the microorganisms commonly employed in the assay of vitamin B_{12}. By means of paper partition chromatography, Winsten and Eigen ('49a, b) developed a method of separating vitamin B_{12} activity from thymidine and other interfering substances. Hoffmann, Stokstad, Hutchings, Dornbush and Jukes ('49) have recently reported a chemical method of correcting for the effect of desoxyribosides. The existence of interfering substances in natural materials was not stressed in the studies reported previously on vitamin B_{12} content ascertained by microbiological assay.

Studies were made, therefore, of the value of applying correction techniques in the determination of vitamin B_{12} content by microbiological assay. The results of these studies are presented in this paper, together with corrected values for the vitamin B_{12} content of several feedstuffs and other nutrients. Evidence is also presented of correlation between the vitamin B_{12} values obtained by microbiological and chick growth assays.

EXPERIMENTAL AND RESULTS

The microbiological assay method of Peeler et al. ('49), using *Lactobacillus leichmannii,* American Type Culture Collection 4797, was used in these experiments. The methods reported by these workers for liberating vitamin B_{12} activity were also used. These involved enzymatic digestion of the animal products with pancreatin under toluene for 20 hours in phosphate buffer at pH 6.7 and 37°C. and digestion of the plant products under toluene with a 50:50 mixture of takadiastase and papain for 40 hours in pH 4.5 acetate buffer at 37°C. Materials already soluble in water were not treated prior to assay.

Comparison of methods of correcting for effect of interfering substances

The paper partition chromatographic method of Winsten and Eigen ('49a, b), as modified by Yacowitz, Norris and Heuser ('49), was used to correct for the effect of the desoxyribosides and other interfering materials. The results of applying these corrective techniques, as measured microbiologically, are presented in table 1. In general, close agreement was shown between the corrected values for the vitamin

TABLE 1

Comparison of methods of correction for interfering substances

MATERIAL	VITAMIN B_{12} CONTENT IN MμG PER GRAM		
	Uncorrected	After chromatography	After alkali [1]
Meat scrap	42	33	36
Homogenized condensed red fish (dry basis)	430	409	416
Red fish meal	96	96	94
Injectable liver extract [2] (15 unit per ml)	1,000	1,000	1,000
Vitamin B_{12} concentrate [3]	8,060	8,060	8,060

[1] Hoffmann et al. ('49).

[2] Expressed in mμg per U.S.P. unit.

[3] Prepared from injectable liver extracts. Expressed in millimicrograms per milliliter.

B_{12} content of the products assayed by the two indirect procedures. The results indicate, therefore, that either method can be used with equal success in correcting for the effect of interfering substances.

Comparison of results obtained with microbiological and chick assays

In order to gain information as to the accuracy of the microbiological assay used in these studies, an experiment

was conducted to determine whether or not the growth response of chicks receiving a purified diet, supplemented with various levels of a vitamin B_{12} concentrate, would correspond to the vitamin B_{12} content of the concentrate as determined by the microbiological assay procedure.

The chicks used in this experiment were hatched from the eggs of hens which were depleted of their body stores of vitamin B_{12}. The method of depletion has been described by Combs et al. ('50). The chicks were placed on an all-vegetable protein basal diet (table 2) for a period of 8 days prior to the experiment in order to deplete them further of vitamin B_{12} stores. At the end of this time 15 chicks were placed in each of the lots. One lot received the basal diet alone; 4 lots received this diet supplemented with graded levels of a vitamin B_{12} concentrate; another lot received 0.5 μg of crystalline B_{12} per 100 gm of diet. The 7th lot received a crude liver paste fraction [5] at 0.2% as a positive control. The vitamin B_{12} concentrate was prepared from an injectable liver extract containing 15 U.S.P. units per milliliter. The liver extract was further purified by the countercurrent distribution technique described by Craig ('44) in order to separate the vitamin B_{12} from unknown chick growth factors. The vitamin B_{12} potency of the final concentrate which was fed to the chicks was assayed by the microbiological procedure and was found to contain 8.06 μg per milliliter. Microbiological assay of the crude liver paste indicated that this material contained 14 μg of vitamin B_{12} per gram.

The average gains of the chicks at the end of the 4th week of the experiment are presented in figure 1. The gain promoted by the crystalline vitamin B_{12}, indicated by "X" in figure 1, was approximately the same as that promoted by an equivalent amount of vitamin B_{12} from the concentrate as determined microbiologically. The response curve presented in figure 1 has been substantiated by other studies using graded levels of crystalline vitamin B_{12}. These studies show that the vitamin B_{12} requirement of chicks having the same

[5] Wilson.

degree of depletion as those used in the present study is approximately 0.9 μg per 100 gm of diet. The results indicate, therefore, that the results obtained by the microbiological

TABLE 2

Composition of basal diet

INGREDIENT	AMOUNT PER 100 LB. OF DIET
Ground yellow corn	36 lb.
Soybean grits, 50% protein [1]	60 lb.
Dicalcium phosphate	2 lb.
Limestone	1 lb.
Iodized salt	0.5 lb.
Cod liver oil (3,000 A — 400 D)	0.5 lb.
$MnSO_4$	15 gm
Riboflavin	113 mg
Vitamin K [2]	400 mg

[1] Fifty per cent low fiber soybean oil meal, Archer-Daniels-Midland Co., Minneapolis, Minn.

[2] Menadione, Merck and Co., Inc., Rahway, New Jersey.

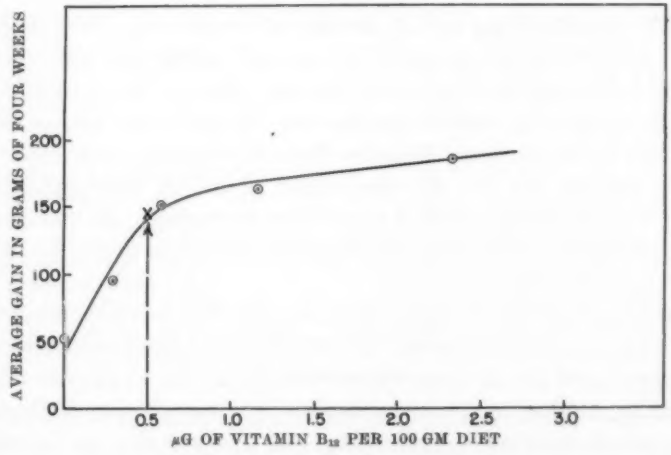

Fig. 1 Comparison of chick growth responses to crystalline and microbiologically determined vitamin B_{12}.

procedure for the vitamin B_{12} content of the materials used are comparable to the results obtained by chick growth assay.

Vitamin B_{12} content of materials

As a consequence of the findings just described, a study of the vitamin B_{12} content of natural feed ingredients and other materials was made by means of the rapid microbiological assay of Peeler et al. ('49) and the correction procedure of Hoffmann et al. ('49).

The vitamin B_{12} content of various materials is presented in table 3. The results of assays of samples of fish meals and fish solubles obtained from different processors show that very little correction was needed. The values presented represent average values from the assay of several samples of the same type of product. The samples assayed varied considerably in potency. The fish products obtained from the Pacific coast processors were, for the most part, more potent in vitamin B_{12} than the products obtained from the Atlantic coast.

The results of assays of samples of meat scrap, liver meal, and miscellaneous products of animal origin are also presented in table 3. The value for the vitamin B_{12} content of meat scrap is a representative one chosen from values obtained for meat scrap samples from 6 different manufacturers. Except for the 40° desiccated, defatted liver product, the liver fractions needed very little correction. A considerable variation in vitamin B_{12} content existed between the two samples of liver L.

Products of plant origin were also studied for their content of vitamin B_{12}. A number of samples of each product were assayed and the average values show that the apparent vitamin B_{12} content of these materials is lowered very little by the alkali treatment, indicating that the activity is, for the most part, due to substances other than vitamin B_{12}, except possibly in the case of leafy plants.

TABLE 3

Vitamin B_{12} content of various materials

MATERIAL	VITAMIN B_{12} CONTENT IN MμG PER GM		
	Uncorrected	After alkali [1]	Corrected
Fish meals and fish solubles:			
Menhaden fish meal with solubles	66	4	62
Red fish meal	96	2	94
Red fish solubles (dry basis)	143	0	143
Sardine fish meal	158	4	154
Sardine solubles (dry basis)	389	8	381
Herring fish meal	263	4	259
Herring solubles (dry basis)	303	4	299
Cod and haddock meal	98	3	95
Miscellaneous animal products:			
Meat scrap	42	6	36
Liver meal	317	7	310
40° desiccated defatted liver (Viobin)	413	69	344
Wilson's 95% alc.-soluble liver paste [2]	34,105	0	34,105
Wilson's liver concentrate paste 1:20 [2]	4,207	0	4,207
Wilson's liver fraction L:			
(sample 1)	389	0	389
(sample 2)	1,337	0	1,337
Crude casein	104	0	104
Various plant products:			
Wheat	7	6	1
Soybean meal	11	9	2
Yellow corn	5	5	0
Oats	10	7	3
Dehydrated alfalfa meal	27	24	3
Dried brewers' yeast	17	16	1
Distillers' dried solubles	8	7.5	0.5

[1] Hoffmann et al. ('49).

[2] Expressed on dry basis.

Studies on the vitamin B_{12} content of alfalfa meal

Alfalfa and other green leafy plants have been reported by Cary and Hartman ('40) to contain an unidentified "factor X" which is required for the normal growth of the rat. Hartman and Cary ('49) have recently found the need for

this unidentified factor to be met by crystalline vitamin B_{12}. In view of these reports, a study was undertaken of the vitamin B_{12} activity of alfalfa and other green leafy plants by assay with *L. leichmannii*.

The results presented in table 3 show that the activity found in alfalfa was not destroyed to any great extent by treatment with alkali according to the method described by Hoffmann et al. ('49). An attempt was made, therefore, to liberate the vitamin B_{12} activity of this product in a form that is destroyed by the alkali treatment. An alfalfa extract, crystalline vitamin B_{12} and thymidine were autoclaved at pH 2.0 for 15

TABLE 4

Effect of acid and alkali treatment of vitamin B_{12}, thymidine and alfalfa extract

SUBSTANCE [1]	PERCENTAGE ACTIVITY DESTROYED		
	Alkali [2]	Acid [3]	Acid and alkali [2,3]
Vitamin B_{12}	100	86.7	100
Thymidine	0	2.5	2.5
Alfalfa extract	11	60.4	60.4

[1] Five milliliter aliquots containing 0.5 mμg of vitamin B_{12}, 25 μg of thymidine and a takadiastase-papain digest extract equivalent to 50 mg of alfalfa meal were subjected to the treatment.

[2] Steam at 100°C. for 30 minutes in 0.2 N NaOH solution. Hoffmann et al. ('49).

[3] Autoclave for 15 minutes at 15 lb. and 120°C. at pH 2.0.

minutes at 15 lb. and 120°C. This treatment was found to cause only a slight decrease in the apparent vitamin B_{12} activity of thymidine. When crystalline vitamin B_{12} and the alfalfa extract were subjected to the same treatment, a considerable amount of destruction occurred. A combination of the acid and alkali treatment resulted in a 100% destruction of vitamin B_{12}, but no further change was observed in the activity of thymidine and the alfalfa extract. The results of this study are presented in table 4.

It appeared, therefore, that at least 60% of the apparent vitamin B_{12} activity of the alfalfa is not due to thymidine.

The presence of certain other desoxyribosides is not ruled out, however, due to the ease with which they are hydrolyzed with mineral acid (Levene and Bass, '31). The possibility exists that the major portion of the activity of the alfalfa may be due to a form of vitamin B_{12} not destroyed by the alkali treatment described by Hoffmann et al. ('49).

In order to obtain a better understanding of the site of the major portion of the apparent vitamin B_{12} activity, resort was made to the paper partition method described by Yaco-

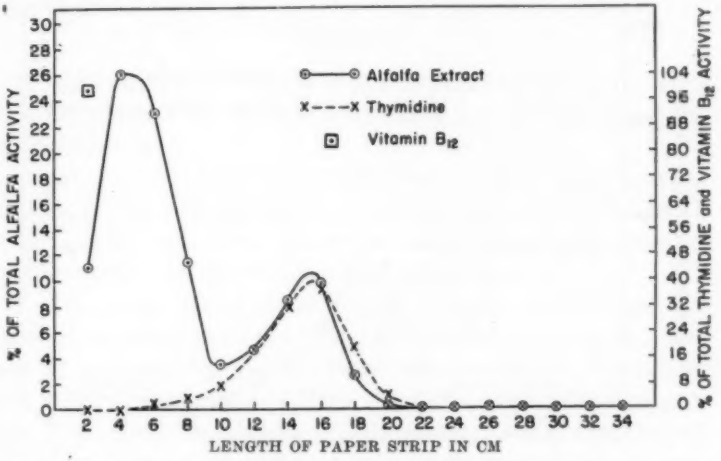
Alfalfa Extract
Thymidine
Vitamin B12
% OF TOTAL ALFALFA ACTIVITY
% OF TOTAL THYMIDINE and VITAMIN B12 ACTIVITY
LENGTH OF PAPER STRIP IN CM

Fig. 2 Response of *L. leichmannii* to *n*-butanol paper partition fractions of alfalfa extract, thymidine and vitamin B_{12}.

witz and others ('49), in which *n*-butyl alcohol saturated with water is used as the mobile solvent and the partition is continued until the solvent front has progressed a total distance of 34 cm. The results of partitioning the alfalfa extract, thymidine and crystalline vitamin B_{12} are presented in figure 2. The crystalline vitamin B_{12} did not move down the chromatogram, whereas the thymidine activity moved so that its R_f value was 0.47. The results are in fairly close agreement with those reported by Winsten and Eigen ('49a) and Smith and Cuthbertson ('49) for the two substances.

The apparent vitamin B_{12} activity of the alfalfa distributed itself into two activity peaks. The peak having the major portion of the activity was concentrated at 4 to 5 cm from the starting line and possessed an R_f value of 0.12 to 0.15. This activity peak was considerably slower moving and its R_f value, therefore, could not be correlated with that of any of the 5 desoxyribosides reported by Smith and Cuthbertson ('49). The lesser peak corresponded almost identically with the thymidine peak and was located 15 to 16 cm from the

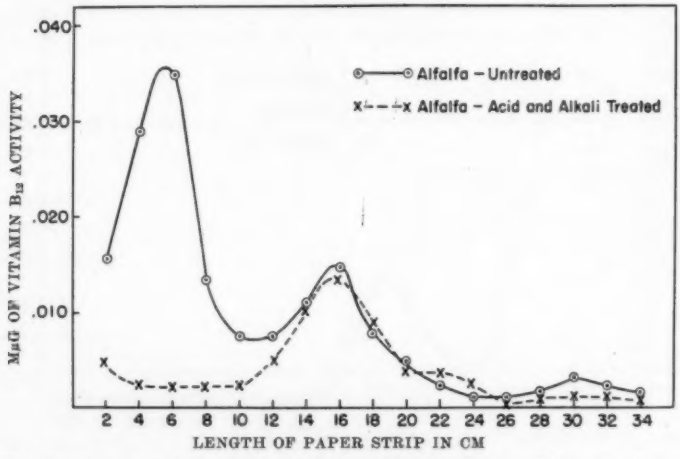
.040
.030
.020
.010
MμG OF VITAMIN B12 ACTIVITY
Alfalfa – Untreated
Alfalfa – Acid and Alkali Treated
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
LENGTH OF PAPER STRIP IN CM

Fig. 3 Response of *L. leichmannii* to *n*-butanol paper partition fractions of alfalfa extract.

starting line, with an average R_f value of 0.46. Whether or not the major peak is identical with one of the slow moving substances reported by Winsten and Eigen ('49a) is not known.

Partition chromatograms were also run on a sample of alfalfa extract, which had been acid- and alkali-treated as described previously, and on a sample of untreated alfalfa extract. The results of this experiment are presented in figure 3. The minor activity peak, which was confined to the thymidine locus, was unaltered by the treatment. The peak

of major activity, however, was almost completely destroyed. The loss of total activity observed in this study agrees closely with the 60% shown in table 4. It seems probable, on the basis of a consideration of all the results, that the apparent vitamin B_{12} activity destroyed by the acid-alkali treatment was limited to a single substance.

Studies are under way to determine if the activity found in the major peak is effective in promoting chick growth when fed under the conditions used in the chick assay for vitamin B_{12}.

SUMMARY

1. Evidence is presented that comparable values for vitamin B_{12} potency are obtained by chick growth and microbiological assays.

2. Studies revealed that substances which replace vitamin B_{12} in the metabolism of *L. leichmannii* can be differentiated equally well with paper partition chromatography or by destruction of the vitamin with alkali.

3. Using the rapid microbiological assay method and the alkali correction procedure, a study of the vitamin B_{12} content of feedstuffs and other materials was made. Fish meal and related products and liver products were found to contain appreciable quantities of vitamin B_{12}, while cereals and their by-products were found to contain very little, if any, vitamin B_{12}.

4. Two vitamin B_{12} activity peaks were obtained by paper partition of alfalfa meal extracts. The smaller, faster moving peak appeared to be due to thymidine. The other, slow moving peak may be due to a form of vitamin B_{12} not destroyed by alkali, as its R_f value did not correlate with that of any of the known active desoxyribosides.

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EVALUATION OF FIVE PARTIALLY PURIFIED PROTEINS BY NITROGEN BALANCE IN MATURE DOGS, INCLUDING A STUDY OF THE ANTITRYPTIC ACTIVITY OF EGG WHITE

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EXPERIMENTAL

To determine further the utilization of 5 of the partially purified proteins previously studied by growth of young dogs (Mabee and Morgan, '51), nitrogen balance studies were carried out using 6 adult stock dogs of the same strain as the young dogs.

The endogenous nitrogen level for each dog was first determined by feeding the non-protein basal diet to provide 80 cal. per day per kilogram of body weight. A second endogenous period was determined toward the end of the series and an average figure for the two periods was used in the calculations. In the case of three dogs whose two endogenous levels differed, a third determination was made, and an average of the three figures was used in the calculations.

When placed on each of the different diets, the dogs were given a three-day adjustment period before the collection was begun. On the 4th day and three succeeding days the feces and urine were collected. The urine and feces of each dog were pooled separately for the 4-day period. The urine was acidified with sulfuric acid, collected under toluene and refrigerated. The nitrogen in the urine was determined by

the micro-Kjeldahl method. The feces, moistened with acidic alcohol, were dried in an electric oven at 100°C., the hair removed, and the samples ground in an electric grinder. The total nitrogen in the feces was determined by the macro-Kjeldahl method.

The proteins used in the study were casein, defatted whole egg powder, dried egg albumin, peanut flour, and dried beef muscle. Wheat gluten was not included in this study, as the supply of the Rutgers gluten was exhausted and the gluten on hand contained 42.1% protein compared with 81% in the Rutgers sample. The casein used was not that from Rutgers but was a commercially available vitamin-free casein [1] which, by previous experiments, had been shown to be comparable with the former in protein efficiency. The defatted egg, egg albumin, peanut flour and beef were all of the Rutgers shipment. The proteins were fed at approximately a 7% level, and all diets were isocaloric. Because the albumin diet caused diarrhea, 10 gm of agar were incorporated into the daily diet allotment. The diarrhea however, was not as severe as that observed when a higher level of the albumin had been fed. The agar made the collection of the feces less difficult and the separation of the urine more reliable On this diet the dogs were given a 5-day adjustment period instead of the usual three-day period.

The nitrogen contents of the protein sources, in per cent, were found to be: casein 14.12, peanut flour 9.25, egg albumin 11.80, whole egg powder 11.80, dried beef muscle 14.41. The diets were made as shown in table 1.

Since the egg albumin fed at both 18 and 40% levels in the growth study with young dogs (Mabee and Morgan, '51) had proved to be highly indigestible, a further experiment was carried out with two adult female dogs, using the 40% egg albumin diet. All procedures were the same as those used for the balance studies with the 7% protein diets. This experiment was made in the hope of discovering whether the

[1] General Biochemicals, Inc.

mature animal was better equipped than the young dog to resist the effect of the antitryptic substance in egg albumin.

Severe diarrhea resulted from feeding the 40% albumin diet and it was necessary to incorporate in the diet celluflour, 10%, and agar, 10 gm daily to overcome the condition.

TABLE 1

Composition of the diets

	BASAL DIET	TEST DIET
	%	%
Test protein	. .	7.0
Salts 5 [1]	2.5	2.5
$CaCO_3$	1.5	1.5
Fat [2]	10.0	10.0
Sucrose	86.0	79.0
	mg %	*mg %*
Thiamine	0.2	0.2
Riboflavin	0.2	0.2
Pyridoxine	0.2	0.2
Folic acid	0.2	0.2
Calcium pantothenate	6.0	6.0
p-Aminobenzoic acid	6.0	6.0
Niacinamide	4.0	4.0
Inositol	50.0	50.0
Choline	100.0	100.0
Biotin [3]	0.01	0.01
Vitamin A	4,600 I.U.[4]	4,600 I.U.[4]
Vitamin D	210 I.U.[4]	210 I.U.[4]

[1] Axelrod, H. E., A. F. Morgan and S. Lepkovsky, J. Biol. Chem., *160:* 155, 1945.
[2] Primex, hydrogenated vegetable fat.
[3] To the egg albumin diet 0.03 mg % biotin was added.
[4] Per dog per day.

The protein-free or basal periods were also repeated with this modified diet. Seven-day adjustment periods were allowed with each change of diet. The diets were made up as shown in table 1 except that 40% egg albumin was incorporated at the expense of a similar amount of sucrose. The actual nitrogen content of the resulting diet was 5.4%, 33.4% protein.

It has been established (Lineweaver and Murray, '47) that the antitryptic activity of egg white is identified with the ovomucoid and that the inhibitory activity is specific for trypsin and destroyed by denaturation of the ovomucoid. Exposure to heat of a water solution at 80°C. for one-half hour at pH 3 to 7 had little effect on the antitrypsin but at pH 9 produced at least 90% destruction of it. In the dry state the antitrypsin is more stable to heat, apparently, for heating at 60°, 110° and 140°C. for 18 hours was required for reductions of 40, 94 and more than 99% of the inhibitory activity.

In the growth study with dogs (Mabee and Morgan, '51) an attempt was made to destroy the trypsin inhibitor present in the egg albumin. This was done by heating a suspension of the daily food ration for one of the dogs in a double boiler to 73°C. Subsequent tests were conducted to determine the effect of this procedure on the trypsin inhibitory potency of the egg albumin. Furthermore, dry samples of this protein were subjected to heat at temperatures varying from 60° to 150°C. for varying periods of time in order to determine the heat lability of the inhibitor in this particular egg white preparation.

The inhibitory effect of the egg albumin samples was determined by a method similar to that outlined by Westfall and Hauge ('47). Our procedure deviated in: (a) the use of crystalline trypsin [2] instead of the crude pancreatic extract; (b) a prolongation of the incubation period by 5 minutes; and (c) determination of the degree of digestion by analysis for free tyrosine in the final mixture with the Folin and Ciocalteu ('29) reagent as described by Anson ('38). Forty micrograms of trypsin per milliliter were found to give a suitable concentration.

With raw dry albumin as the substance of reference for the inhibitory potency, the suitable concentration for maximum inhibition was determined. When increasing concentrations of egg albumin were plotted against the logarithm

[2] Crystalline trypsin, Armour.

of the tyrosine value, a linear relationship was found up to a concentration of 0.02% egg albumin. This level was chosen in all subsequent experiments.

In order to duplicate the heating procedure used in preparing the diet for feeding one of the young dogs, a mixture of the diet and water was heated in a water bath of 84° till the mixture reached the temperature of 73°C. The tubes were either removed and cooled or held at 73°C for 10 minutes. A homogeneous mixture necessary for uniform digestion was obtained by the use of a blender without loss of the inhibitory potency as a result of the beating (table 2). This conforms with the finding of Rose and MacLeod ('23) that

TABLE 2

Effect of beating in a blender on antitryptic activity of dried egg albumin

SAMPLE AT 0.02% LEVEL	UNITS OF INHIBITOR [1]
Raw egg albumin	100
Raw egg albumin, beaten	102 ± 10
40% albumin diet, unheated	85 ± 10
40% albumin diet, unheated, beaten	100 ± 10

[1] Average of 6 determinations.

beaten raw egg white was no more digestible by young women subjects than the unbeaten white. The belief that the beating of raw egg or egg white in eggnogs is a practical means of decreasing the antitryptic property of the egg white is apparently without foundation.

RESULTS AND DISCUSSION

The data for all periods for each of the 6 dogs fed the 7% protein diets are shown in table 3. Although there was some spread in the values obtained, it is clear, as is shown in the summary (table 4), that the egg albumin and the defatted whole egg were superior to the other proteins for maintenance of nitrogen balance in normal young adult dogs. Casein was

TABLE 3

Utilization of nitrogen of 6 partially purified proteins by young adult dogs, in mg/kg/day

PERIOD	DOG NO.	WEIGHT	PROTEIN FED	N INTAKE	URINE N	FECAL N	BALANCE N	ABSORBED N	RETAINED N	TRUE DIGESTIBILITY	BIOL. VALUE
		kg								%	
1,6	38 ♀	7.3	None	None	139	22	−161				
2		6.9	Casein	210	203	28	−21	204	140	97	68
3		6.9	Beef muscle	202	233	25	−56	199	105	98	53
4		6.9	Defatted whole egg	213	149	30	+34	205	195	96	95
5		7.2	Peanut flour	200	220	41	−61	181	101	91	55
7		6.7	Egg albumin	216	170	69	−23	170	139	79	82
1,6	39 ♀	6.6	None	None	134	20	−154				
2		6.3	Casein	200	216	25	−41	195	113	98	58
3		6.1	Beef muscle	210	228	35	−53	195	101	93	52
4		6.3	Def. wh. egg	213	168	31	+14	202	168	95	83
5		6.5	Peanut flour	200	202	35	−37	185	117	93	63
7		6.4	Egg albumin	213	156	49	+8	184	162	86	88
1,6	40 ♂	7.5	None	None	136	20	−156				
2		7.3	Casein	214	206	25	−17	209	139	98	66
4		7.0	Def. wh. egg	212	174	27	+11	205	167	97	81
5		7.1	Peanut flour	200	238	30	−68	190	88	95	46
1,6,8	41 ♂	7.7	None	None	143	16	−159				
2		7.6	Casein	220	198	24	−2	212	163	97	77
3		7.2	Beef muscle	205	212	19	−26	202	161	98	65
4		7.7	Def. wh. egg	213	166	26	+21	203	180	95	88
5		7.9	Peanut flour	200	191	36	−27	180	132	90	73
7		7.0	Egg albumin	215	143	48	+24	183	183	85	100
1,6,8	42 ♂	7.9	None	None	132	20	−152				
2		8.2	Casein	210	182	18	+10	212	162	101	76
3		7.8	Beef muscle	200	213	19	−32	201	120	100	60
5		8.4	Peanut flour	199	198	28	−27	191	125	96	65
7		7.9	Egg albumin	213	170	42	+1	191	153	90	80
1,6,8	43 ♀	6.8	None	None	166	17	−183				
2		7.0	Casein	214	243	21	−50	210	133	98	63
3		7.0	Beef muscle	206	262	18	−74	205	109	100	53
4		6.9	Def. wh. egg	214	192	25	−3	206	180	96	87
7		6.3	Egg albumin	213	175	57	−19	173	164	81	95

next in biological value, with peanut flour and beef muscle following in that order. The beef was of lowest value in all cases, a surprising finding in view of its excellent value for the growth of young dogs. A similar discrepancy was noted by Morgan and Kern ('34) in experiments with rats some years ago. The explanation may lie in the preponderance of a demand for amino acids for muscle and organ production during growth and for hair and secreted protein in maturity,

TABLE 4

Averages of N exchange of 5 or 6 dogs fed 6 partially purified proteins at 7% level, in mg N/kg/day

DIET	CASEIN	BEEF	WHOLE EGG	PEANUT FLOUR	EGG ALBUMIN
Test protein eaten	208	205	213	200	214
Test protein fecal N	24	23	28	34	53
No protein fecal N	19	19	19	20	19
Test protein waste fecal N	5	4	9	14	34
Absorbed N	203	201	204	186	180
True digestibility, %	97	98	96	93	84
N balance	−24	−48	+15	−44	−2
Test protein urine N	208	230	170	210	163
No protein urine N	143	143	143	137	143
Test protein waste urine N	65	87	27	73	20
Retained N	138	114	177	113	160
Biological value	68	57	86	61	89

although for the latter purpose the relatively high cystine-methionine content of beef muscle protein might be expected to render it effective. The deficiency must lie elsewhere.

The change in position of the egg albumin from lowest in growth to highest in maintenance value (table 5) was due largely to the lower protein level used in the balance experiment, with consequent decrease in the amount of antitrypsin in the diet. There was little improvement in the capacity of the mature dogs to denature the antitrypsin, since the apparent digestibility of the 40% albumin diet was 53% in the mature dogs (table 6) and 49% in the young growing animals. It is usually assumed that the antitrypsin of egg white

is completely denatured by the gastric hydrochloric acid. In spite of the supposedly high acidity of the dog's gastric secretion, relatively little denaturation occurred in the digestive tracts of the dogs of this colony.

With 7% albumin in the diet the true digestibility of the protein was found to be 84% (table 4), as compared with

TABLE 5

Growth and maintenance values of proteins for dogs

PROTEINS	RELATIVE VALUE FOR GROWTH	RELATIVE VALUE FOR MAINTENANCE
Whole egg	100	100
Egg albumin	35	103
Casein	99	79
Peanut flour	56	71
Beef muscle	101	66
Gluten	46	

TABLE 6

The true digestibility of dried unheated egg albumin fed at a 40% level to mature dogs, in mg/kg/day

DOG	WEIGHT	PROTEIN FED	N INTAKE	URINE N	FECAL N	BALANCE N	ABSORBED N	TRUE DIGESTIBILITY
	kg							%
44 ♀	9.0	None	1	106	17	— 122		
	9.4	Egg albumin	1,000	245	485	+ 270	532	53
45 ♀	13.0	None	1	133	16	— 148		
	13.2	Egg albumin	1,000	375	461	+ 164	555	55

96% for whole egg. The reduction, about 12%, may be compared with the 42% reduction in digestibility seen with the 40% albumin diet (table 5). Thus, in mature dogs a roughly linear relationship appeared to exist between the amount of inhibitor in the diet and the loss of digestibility of the protein.

The results shown in table 7 on the effect of heating the diet upon the antitrypsin of the egg white represent the average of 6 samples from three different series of experiments. Since the results varied from day to day as much as 10%, the differences do not seem significant. Under the conditions of this experiment the inhibitory potency of the egg albumin present in the diet was undiminished due to heating at 73°C. for this length of time.

The inhibitory factor was also rather stable to heat when samples of egg albumin in the dry state were subjected to temperatures of from 80° to 150°C. for periods of from one to 18 hours (table 8). No significant lowering of inhibitory

TABLE 7

Effect of heating to 73° on antitryptic activity of dried egg albumin

SAMPLE	TREATMENT	UNITS OF INHIBITOR
Egg albumin	unheated	100
40% albumin diet	unheated	100 ± 8
40% albumin diet	heated to 73° in 5 min.	93 ± 7
40% albumin diet	heated to 73° in 5 min. and held 10 min.	86 ± 10

potency was observed except in samples heated to 150°. The inactivation of the inhibitor progressed somewhat slowly with time.

It is evident that very little change in the antitryptic quality of the egg albumin followed the heating of a suspension of the diet at 73°C. for 10 minutes. Nevertheless some improvement was seen in the condition of the young dog fed this preparation (Mabee and Morgan, '51). The antitrypsin of this sample of egg albumin was more stable to dry heat than the samples used by Lineweaver and Murray ('47), since exposure to 80° and 100° for 18 hours caused only 4 and 6% reductions in inhibitory activity instead of the 40 and 94% reductions reported by the former authors. Exposure to 150° for 18

hours produced complete destruction of inhibition in both studies. This conforms with the findings of Mecham and Olcott ('47) that heating proteins for 18 hours at 110° to 153° caused little change except in solubility, but at and above 153° extensive decomposition occurred. Complete denaturation of the antitryptic protein may be necessary to prevent the inhibition. Simultaneous decomposition of the other proteins of the egg albumin may be expected to occur under these conditions.

TABLE 8

Diminution of antitryptic activity of dried egg albumin by heat treatment

SAMPLE	TREATMENT	UNITS OF INHIBITOR
Egg albumin	Unheated	100
Egg albumin	Heated at 80°C.	
	2 hours	98
	4½ hours	105
	18 hours	96
Egg albumin	Heated at 100°C.	
	1 hour	110
	2 hours	111
	18 hours	94
Egg albumin	Heated at 150°C.	
	1 hour	41
	2 hours	27
	18 hours	0

These results cast doubt on the desirability of incorporating egg white in any form into dog foods. Since long continued heating at high temperatures is required for the destruction of the antitrypsin, and since concomitant denaturation of the other proteins of the egg white is likely to occur, little value can be ascribed to egg white in canine nutrition. The situation is apparently quite different with respect to the rat (Ruegamer et al., '50; Mitchell and Beadles, '50) and the human (Hawley et al., '48), in which species complete utilization of the egg albumin was observed.

The experiments of Bateman ('16) with dogs, cats, rabbits and men indicated only 50 to 75% digestibility of raw egg white and diarrhea usually resulted when appreciable amounts were ingested.

A recent study of the utilization of raw and heated egg white using two men as subjects (Scudamore et al., '49) indicated no depression of nitrogen balance when this protein contributed 40% of the 50-gm daily protein intake used. The total daily calorie intake was 2,500 and the contribution made by the egg white to the total diet was therefore about 3.2%. The absorption of the unheated egg white was 68 to 77% in the two subjects, as compared with 73 and 80% for unheated lactalbumin. The decrease was thus 5 and 3%, figures which are fairly comparable with the 12% decrease shown by our dogs which received unheated egg white at a 7% calorie level. The heated egg white, wet autoclaved, temperature and time not stated, both without and with 4 gm ovomucoid was better absorbed than the lactalbumin. Obviously, this processing was effective for the destruction of antitryptic activity for these human subjects.

The long-assumed superiority of meat protein in the dog's diet may also well be questioned with reference to the maintenance of adult animals. Both casein and peanut flour allowed slightly but significantly better nitrogen utilization in these experiments than did beef muscle. This could hardly be due to any damage incurred in the preparation of the beef powder, since its growth value was found to be of a high order.

SUMMARY

Five partially purified proteins which had been previously evaluated for efficiency in promoting the growth of cocker spaniel dogs were examined by the nitrogen balance method, with 6 young adult dogs of the same strain as subjects. At a 7% level in the diet the true digestibilities were: beef muscle 98, casein 97, whole egg 96, peanut flour 93, egg albumin 84. The biological values were: egg albumin 89, whole egg 86, casein 68, peanut flour 61, beef muscle 57.

At a 40% level in the diet the true digestibility of the egg albumin, tested with two fully mature dogs, was found to be 54%. Since in the young dogs the corresponding value was 49%, no improvement with age in ability to denature the antitrypsin of egg white can be said to have occurred.

Vigorous beating in a blender of egg white suspensions did not decrease their antitryptic activity, nor did heating the suspensions at 73° for 10 minutes. Dry heating at 80° or 100°C. for two to 18 hours produced insignificant changes in the antitryptic activity, but heating at 150° for one, two and 18 hours destroyed 59, 73 and 100% of this activity. Complete denaturation and partial decomposition of the antitryptic protein appeared necessary for prevention of the inhibitory effect.

The vulnerability of the dog to the antitryptic action of egg white is contrasted with the excellent utilization of this protein by young and adult rats and by adult humans.

The low value of beef muscle protein for maintenance of adult dogs as compared with its superior value for growth may be due to changes in amino acid requirements from those preponderantly for muscle and organ growth to those for protein in hair and secretions.

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THE USE OF CHROMIC OXIDE AS AN INDEX MATERIAL IN DIGESTION TRIALS WITH HUMAN SUBJECTS [1]

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ONE FIGURE

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One of the major problems associated with the technique of digestibility studies employing either "time" or "marker" collection of feces is that of accurately identifying the residues of the test diet. This difficulty can be eliminated by estimating digestibility from the ratios to feed and to feces of an index substance incorporated in the diet, provided it is nutritionally inert and subsequently eliminated quantitatively in the feces.

Numerous investigations have been conducted on the appropriateness of the use of chromic oxide as such an index material in digestibility studies with farm animals. Except for two publications of Kreula on carotene absorption, no reports have been found in the literature regarding the use of this substance in human studies. Kreula ('47) concluded that "it gives values completely in accordance with those obtained by the method based on the quantitative collection of feces." In 1950 he reaffirmed his earlier conclusions and added that the Cr_2O_3 did not show any detrimental influence on the health of the subjects.

[1] Contribution from the Faculty of Agriculture, McGill University, MacDonald College, Quebec, Canada. Journal Series no. 270.

The success of the method with any species of animal depends on whether the index material will pass unchanged and unabsorbed through the intestinal tract and be uniformly mixed with the feces. With some animals this may be achieved by thoroughly mixing the index material with the feed. In the case of man, the nature of the diet is such that it is difficult to provide an even distribution of any one dietary component throughout the food intake. No studies have been made of the effects of the method of administration of the index (Cr_2O_3) on the time or regularity of its excretion or its distribution in the stool.

Therefore a series of tests was undertaken in this laboratory with human subjects in order to obtain data on the rate of the passage of Cr_2O_3 through the digestive tract and the uniformity of its distribution in the excrement according to whether the day's intake was taken at one time, in three equal portions, or in fixed ratio to the dry matter at each feeding.

EXPERIMENTAL PROCEDURE

Preliminary to the main study, three tests, each of 8 days' duration, were conducted with one human subject. In the first test, 1 gm of chromic oxide was administered daily with breakfast. In the second the index was fed in fixed amounts (0.3 gm) thrice daily (at each regular feeding). In the third test chromic oxide was taken with each feeding in proportion to the dry matter consumed (0.25% of the dry matter). The chromic oxide was eaten dry or mixed with sugar.

A basal diet consisting essentially of shredded wheat was fed in all three tests. Approximately 425 gm dry matter were consumed daily. In each trial, chromic oxide administration began on the second day and continued for 4 days. The basal diet was fed for an additional three days after the chromic oxide ingestion had ceased.

Collection of feces began with the first elimination after the intake of the chromic oxide and continued for 7 days. In

order to test the uniformity of chromic oxide distribution in the feces, each defecation was arbitrarily subdivided into two, or, if sufficient in quantity, into three portions, which were dried separately for analysis. A carmine marker was taken on the morning following the last day of chromic oxide administration. For the conventional marker-to-marker method of determining digestibility, records were kept of the total feces excreted from the appearance of Cr_2O_3 up to, but not including, the appearance of the carmine, an average of 7 days.

The dried feces samples were analysed for Cr_2O_3 by the method developed in this laboratory (Schürch, Lloyd and Crampton, '50). Apparent digestion coefficients for dietary dry matter were calculated for each of the 47 samples available for the index method, using the formula:

$$\% \text{ digestibility of nutrient} = \frac{100(a-b)}{a}$$

where a = parts of nutrient (e.g., dry matter, protein, ether extract, etc.) per unit index material in the food, and b = parts of nutrient per unit index material in the feces.

RESULTS

Tests 1–3 inclusive

The results of the first three tests are shown in table 1.

The average digestion coefficient obtained by the index method for the three 4-day collection periods (89.0) is in good agreement with that obtained by the conventional method (88.3). Comparison of the digestion coefficients calculated for each sub-sample indicate that the chromic oxide when administered once daily, as in test 1, did not appear to be evacuated as evenly as when fed thrice daily (test 2) or in proportion to the dry matter intake (test 3).

On the second and third tests the index was excreted uniformly in proportion to the dry matter of the feces beginning with the second defecation following its first administration. All of the fecal Cr_2O_3 appeared to have been passed within

TABLE 1

Dry matter digestibility by conventional quantitative marker-to-marker method versus the chromic oxide index method, according to three schedules of administration of Cr_2O_3

METHOD	DAYS	DEFECATIONS: Within days	DEFECATIONS: Stool[1] sub-sample	DIGESTION COEFFICIENTS: Test 1 — Chromic oxide ingested once daily (1 gm)	Test 2 — Chromic oxide ingested thrice daily in equal portions of 0.3 gm	Test 3 — Chromic oxide ingested at each feeding in fixed proportion to dry matter intake
				%	%	%
Conventional quantiative collection	7 (approx.)	–	–	91	88	89
		1	a	92	88	82
			b	92	86	87
			c	88	87	86
		2	a	83	88	89
	2nd		b	83	88	
			c	82	88	
		3	a	92		
			b	92		
			c	91		
Chromic oxide method		1	a	87	89	90
			b	86	90	90
	3rd		c	85	90	
		2	a			90
			b			90
		1	a	90	89	89
	4th		b	91	89	88
			c	91	89	88
		1	a	92	88	90
			b	92	87	89
	5th		c	92	87	89
		2	a	89		90
Means (Cr_2O_3 method)				90	88	89

[1] Each stool was arbitrarily sub-sampled in order to test the uniformity of chromic oxide distribution.

48 hours after the last ingestion, with a recovery in the three tests of 95, 102 and 103%, respectively. These data are shown graphically in figure 1.

A statistical analysis of the data showed no significant differences among the digestion coefficients obtained with any of the samples in any of the tests.

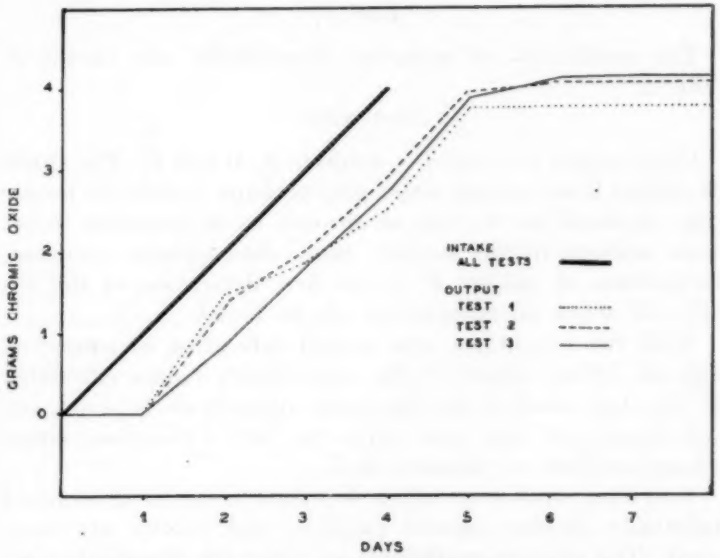

Fig. 1 Chromic oxide intake and fecal excretion. Test 1 — chromic oxide ingested once daily; test 2 — chromic oxide ingested thrice daily; test 3 — chromic oxide ingested with every feeding at the rate of 0.25% of the dry matter intake.

Based on the results of these three preliminary tests, and in conjunction with another series of digestion trials, a 4th test was run, using 6 subjects. The chromic oxide was administered with each feeding at the rate of 0.25% of the dry matter intake, as in test 3. The basal diet consisted essentially of shredded wheat and of ground wheat baked into muffins, supplemented with fruit juice, cream, sugar and methylcellu-

lose. At the conclusion of the 4-day test period, the subjects resumed their normal diets. The same procedure with regard to collection and subdivision of feces samples used in the previous tests was employed in this trial. Digestibility values were calculated by the conventional quantitative collection method for each subject and by the chromic oxide method for each stool sub-sample.

Test 4

The coefficients of apparent digestibility are shown in table 2.

DISCUSSION

Constipation was noted in subjects A, D and E. The stools of subject E were small, which may perhaps explain the longer time required for the rate of chromic oxide excretion to become uniform in this subject. Some discrepancies occurred, in the case of subject F, in the first defecation of the 5th day, for which no explanation can be found.

With two exceptions (the second defecation of subject E and the 7th of subject F) the digestibility values calculated in this test showed no significant differences between any sub-samples of any stool after the first defecation, either among subjects or between days.

Nutrition studies in which digestion is to be determined habitually involve several subjects, and results are averaged. The average coefficients of apparent digestibility obtained in the present study, assembled by defecations, are shown in table 3.

These data show that the index procedure, when applied to defecations subsequent to the first index administration, resulted in digestion coefficients about 3% lower than those determined by a 5-day marker-to-marker collection method. In as much as the index was distributed uniformly within stools, one might be led to suspect that the discrepancies did not lie in the Cr_2O_3 values. There is no theoretical reason why the marker-to-marker values might not be in error. For example, at the end of each experimental diet feeding pe-

TABLE 2

Dry matter digestibility by conventional quantitative marker-to-marker method versus the chromic oxide index method (6 subjects)

METHOD	DAYS	DEFECATIONS		DIGESTION COEFFICIENTS					
		Within days	Stool [1] sub-sample	Subject A	Subject B	Subject C	Subject D	Subject E	Subject F
				%	%	%	%	%	%
Quantitative collection	5 (approx.)	–	–	92	90	90	89	89	90
		1	a		84	79			78
			b		86	86			82
			c			87			84
	2nd								
		2	a		88	88			
			b		87	86			
			c		87	87			
		1	a	x [4]	91	91	79	78	86
			b	x	91	91		88	86
			c	78		90			
	3rd								
Chromic oxide (administered with each feeding at the rate of 0.25% of the dry matter of the diet)		2	a			90		83	90
			b			89		89	90
			c						90
		1	a	90	90	89	88	89	88
			b	90	90	91			88
			c	90	90	91			
	4th [2]	2	a		90		89	86	87
			b					86	86
		3	a		87		89	84	87
			b		88		89	86	87
		1	a	88	88	[3]	86	87	87
			b	88	87			87	85
			c	88					83
	5th [2]								
		2	a			86	86		85
			b			86			87
			c			86			
Means (Cr_2O_3 method, omitting first defecation following Cr_2O_3 intake)				89	89	89	88	86	87

[1] Each stool was arbitrarily sub-sampled to test the uniformity of chromic oxide distribution.

[2] Last chromic oxide administration.

[3] Sub-sampling for chromic oxide determination was terminated at noon of the 5th day.

[4] No chromium in samples.

[5] Sub-samples lost.

riod not only was a carmine marker fed, but the diet was changed enough in nature so that there was a detectable difference in feces character. It was evident from examination of the stools that the carmine marker did not exactly separate the last test foods from the subsequent diet. Thus some feces were discarded which, according to physical character, were from the last test meal, although they were marked with carmine.

Such an error can be minimized by longer collection periods, as is evident from the agreement between the 7-day

TABLE 3

Coefficients of apparent digestibility of the dry matter of the diet determined by the index method, arranged in order of defecation following initial Cr_2O_3 intake

DEFECATIONS AFTER START OF Cr_2O_3 INTAKE	SUBJECTS						AVERAGE
	A	B	C	D	E	F	
	%	%	%	%	%	%	%
1st	78	85	84	79	83	81	82
2nd	90	87	87	88	86	86	87
3rd	88	91	91	89	89	90	90
4th		90	90	89	86	86	88
5th		90	89	86	85	86	87
6th		88		86	87	87	87
7th		88	86			85	86
8th						86	86
Means							
(Cr_2O_3 method, omitting first defecation)							87.3
Conventional marker-to-marker method							90.0

marker-to-marker coefficients and those obtained by the index method in tests 1, 2 and 3. In human studies, however, it is not always practicable, and is often impossible, to arrange for collection periods of "optimum" length.

The results shown in this study indicate that where several subjects are involved composite samples representing the major portion of each of 4 successive defecations, beginning with the second following the ingestion of the chromic oxide, will suffice for estimation of the apparent digestibility of the diet or dietary components under study.

CONCLUSIONS

Chromic oxide (chromium sesquioxide, chrome green, Cr_2O_3), when ingested at each regular feeding and in amounts comprising 0.25% of the dry matter of the food eaten, is a satisfactory index substance for use in digestibility studies of the human diet.

The first defecation following the Cr_2O_3 ingestion is unreliable, but subsequently the index is voided mixed uniformly with the feces as long as it continues to be fed.

A composite from each individual on test, consisting of the major portion of the second to the 5th defecations, inclusive, after the first ingestion of the index, is adequate to determine the coefficient of apparent digestibility of those dietary components to which the test for digestibility is applicable.

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THE METABOLISM OF COBALT IN LAMBS

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Several research workers have suggested that cobalt functions in the ruminant animal primarily through some mechanism in the rumen, probably one related to the activities of the microflora. This hypothesis initially stemmed from two pieces of evidence. First, only ruminants require cobalt. At least the natural or experimental occurrence of cobalt deficiency has been reported only in cattle and sheep. Horses and swine have not been shown to be affected by low cobalt rations and laboratory efforts to produce cobalt deficiency have failed with the rat (Houk et al., '46) and with the rabbit and guinea pig (Thompson and Ellis, '47). Apparently, if non-ruminants require cobalt, it is needed in extremely minute quantities. Second, only orally administered cobalt is therapeutically effective in cobalt deficiency. Parenterally administered cobalt is not beneficial, which suggests that the element must play some important role in the alimentary tract. The ineffectiveness of parenterally administered cobalt was first mentioned by McCance and Widdowson ('44) and has been confirmed by Smith et al. ('50).

Recently, Gall et al. ('49) have reported studies which indicate that the ruminal bacteria are directly influenced by cobalt intakes. Also, Tosic and Mitchell ('48) observed that rumen

[1] This paper is a portion of a study conducted by this author in partial fulfillment of the requirements for the Ph.D. degree from Cornell University.

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[3] The authors gratefully acknowledge the aid of Dr. K. C. Beeson of the U. S. Plant, Soils, and Nutrition Laboratory for cobalt analyses, and Dr. L. C. Norris and Dr. H. T. Peeler for the fractions of liver extract.

microorganisms selectively take up orally administered cobalt. There is, therefore, evidence relating cobalt to the rumen microorganisms but the metabolic function or functions are still unknown. Ray et al. ('48) theorized that cobalt may be essential for the snythesis of B vitamins by the ruminal organisms; however, they failed to obtain a conclusive response in cobalt-deficient lambs by treatment with various B vitamins. With the report of the identity of vitamin B_{12} (Rickes et al., '48), which contains cobalt, it was hypothesized that cobalt deficiency is not a lack of cobalt per se, but a lack of vitamin B_{12}. Becker et al. ('49) could not confirm this belief, since oral or intramuscular administration of vitamin B_{12} was of no therapeutic value for cobalt-deficient lambs. Evidence that some factor in liver has therapeutic activity was presented by Filmer ('33).

The studies reported herein were conducted to uncover some metabolic functions of cobalt in ruminant nutrition by (1) determining the influence of cobalt deficiency upon the ability of lambs to digest the various nutrients of the ration, and (2) investigating the therapeutic value of some biological products possibly related to cobalt deficiency.

EXPERIMENTAL

Experiment I — Digestibility studies

Two groups of western wether lambs, each group composed of 6 animals, were used for this study. All lambs had previously been on a cobalt-deficient ration consisting of low-cobalt hay (mixed grasses), powdered whole milk,[4] shelled yellow corn, and common salt fed since December 18, 1947. By July 9, 1948, the entire group showed evidence of cobalt deficiency as measured by appetite, weight gains, general appearance, and the hemoglobin level of the blood. At this time the lambs of the positive control group were fed 1 mg of cobalt per day in the form of $CoCl_2 \cdot 6H_2O$. The remaining 6 lambs were continued on the deficient ration alone. The digestion trials were

[4] Borden's Klim.

initiated after the positive control group had received cobalt for at least 5 weeks and were well on the road to recovery. The details of growth, feed intake and blood analyses have been reported elsewhere (Smith et al., '50).

Each collection period consisted of 10 days, which were preceded by a three-day preliminary period of adjustment to the new environment. A three-day preliminary period was considered adequate since there was no change in the ration fed. During the feeding period a ration consisting of equal parts by weight of concentrates and roughage was offered. However, in the case of the cobalt-deficient group, appetite was too erratic to permit establishment of a constant feed intake or proportion of ingredients during the trial. The chemical composition of the feeds used is given in table 1.

TABLE 1

Average chemical composition of the feedstuffs fed during the digestibility trials

FEEDSTUFFS	DRY MATTER	CRUDE PROTEIN	CRUDE FAT	CRUDE FIBER	N-FREE EXTRACT	ASH	COBALT
	%	%	%	%	%	%	*p.p.m.*
Shelled yellow corn	86.5	8.82	4.6	2.1	69.6	1.36	0.05
Powdered whole milk	95.2	25.06	7.8	0.2	56.4	5.85	< 0.01
Grass hay	90.2	5.94	1.4	36.5	43.1	3.75	0.01–0.02

Samples of feeds, orts, and feces were collected and stored under refrigeration until the termination of the trial, at which time proximate chemical analysis was conducted according to the methods of the Association of Official Agricultural Chemists ('45).

Results of experiment I. The coefficients of apparent digestibility of the various classes of nutrients for cobalt-deficient and cobalt-fed lambs are presented in table 2.

The coefficients of apparent digestibility for the deficient lambs were lower for all the organic nutrients except crude

fiber. Actually, the cobalt-deficient lambs digested the fibrous fraction of their ration more efficiently than the lambs receiving cobalt supplementation. The mean apparent digestibility of the crude fiber was 7.9% higher for the deficient group. An analysis of the data, using Student's "t" test, showed that this difference was significant (19:1). However, cobalt-fed sheep were more efficient in digesting the ether extract and the nitrogen-free extract fractions of the ration. The coefficients of apparent digestibility were signfiicantly different (odds 99:1 and 19:1, respectively) for crude fat and nitrogen-free extract.

TABLE 2

Coefficients of apparent digestibility of rations fed to cobalt-deficient and cobalt-supplemented sheep

(The tabular data are means with their standard errors)

NUTRIENT	COBALT-DEFICIENT	COBALT-SUPPLEMENTED
	%	%
Dry matter	58 ± 2.4	64 ± 1.4
Crude protein	46 ± 4.4	56 ± 1.6
Ether extract	56 ± 3.6 [1]	69 ± 1.6
Crude fiber	49 ± 1.2 [2]	40 ± 3.0
Nitrogen-free extract	67 ± 2.6 [2]	75 ± 1.2

[1] Difference significant at odds of 99:1.
[2] Difference significant at odds of 19:1.

The digestibility of the dry matter and crude protein were not statistically different.

During the trial the average body weight of the deficient lambs was 50.7 lb., whereas the supplemented group averaged 71.2 lb. Furthermore, the deficient group exhibited no gain during the collection period, but the supplemented lambs showed an average increase in body weight of 1.25 lb. for the 10-day period. This indicates clearly that the deficient lambs were typical cases of this nutritional disease.

Data on the level of feed and total digestible nutrient (TDN) consumption of the two groups of lambs are presented in table

3. Only 85 gm of concentrates were consumed daily by the deficient lambs, whereas the cobalt-fed group consumed 418 gm of concentrates daily. For this reason, roughage intake constituted 50.2% of the total feed consumption of the supplemented lambs but the same figure for the deficient group was 75.7%. It is thus apparent that the former group consumed a ration which was more digestible. However, the varying level of daily dry matter consumption by the two groups of lambs is the primary factor responsible for the difference in

TABLE 3

Summary of the feed, dry matter, and total digestible nutrient (TDN) intake of cobalt-deficient and cobalt-supplemented lambs

(The tabular data are means with their standard errors)

ITEM	COBALT-DEFICIENT	COBALT-SUPPLEMENTED
	gm	*gm*
Average daily feed		
Ground yellow corn	58 ± 14.4	359 ± 18.6
Powdered whole milk	27 ± 6.0	59 ± 0.8
Grass hay	264 ± 13.4	422 ± 40.4
Dry matter consumed daily, per cwt.	622 ± 44.5	1042 ± 73.4
TDN consumed daily, per cwt.	365 ± 29.6	688 ± 48.9

the TDN consumption per centiweight of body weight per day. It cannot be attributed to differences in ration digestibility, since these figures were not found to be significantly different. On an equivalent body weight basis the deficient group consumed only 53% as much TDN as the supplemented lambs.

Experiment II — Investigation of various biological products

Phase A of this study was conducted to determine the curative value of pteroylglutamic acid, simultaneous injection of vitamin B_{12} and cobalt, and a commercial liver extract [5] for

[5] Lilly.

cobalt-deficient lambs. Oral administration and subcutaneous injection of the liver extract were compared for their curative value.

Fifteen wether lambs were available for this experiment. The lambs had been fed a cobalt-deficient ration the same as that employed in experiment I for the previous 8 months. Shelled yellow corn (0.05 p.p.m. of Co), powdered whole milk (0.01 p.p.m. of Co), and grass hay (0.05 p.p.m. of Co) were the feedstuffs used to formulate a ration containing approximately 10% crude protein and consisting of equal parts of concentrates and roughage. At the time the lambs were allotted to the treatments, they clearly exhibited anorexia, emaciation, and depressed hemoglobin level. The groups were treated as follows:

Group 1a. Negative control.

Group 2a. Subcutaneous injection of 10 mg of pteroylglutamic acid per lamb daily.

Group 3a. Subcutaneous injection of 1 μg of vitamin B_{12} and 1 mg of cobalt ($CoCl_2 \cdot 6H_2O$) per lamb daily.

Group 4a. Subcutaneous injection of 1 ml or 15 injectable U.S.P. units of liver extract per lamb daily.

Group 5a. Oral administration of 1 ml or 15 injectable U.S.P. units of liver extract per lamb daily.

Group 6a. Oral administration of 1 mg of cobalt ($CoCl_2 \cdot 6H_2O$) per lamb daily.

All treatments were given twice weekly.

The pteroylglutamic acid used in this experiment was prepared by dissolving the crystalline product in a weak solution of sodium hydroxide.

Vitamin B_{12} was injected either as a solution of the crystalline product or as a commercial concentrate [6] containing 15 μg of B_{12} per milliliter. Each lamb in group 3a received 8 μg of vitamin B_{12} in crystalline form [7] and 30 μg in the form of the commercial concentrate during the 35-day period.

The solution of liver extract used is prepared commercially [8] from fresh whole liver. The particular preparation employed

[6] Rubramin. E. R. Squibb and Sons, New York, N. Y.

[7] Supplied through the courtesy of Merck and Company.

[8] See footnote 5, page 91.

contained 15 injectable U.S.P. units per milliliter and 1.75 p.p.m. of cobalt (dry basis).

During the trial weight records and daily feed records were maintained on all lambs. Hemoglobin was determined at weekly intervals by the method of Sheard and Sanford ('29).

Following the termination of the above test, phase B was conducted in an attempt to concentrate the therapeutically active fraction of the liver extract. Eight wether lambs which had failed to respond to their treatment in phase A were used for this test. All the lambs were considered to be in a severe state of cobalt deficiency. The lambs were allotted into three groups which were treated twice weekly as follows:

Group 1b. Subcutaneous injection of 1 ml of an acid-soluble fraction[9] of the liver extract per lamb daily.

Group 2b. Subcutaneous injection of 2 μg of vitamin B_{12} concentrated from liver extract[10] per lamb daily.

Group 3b. Same as group 4a, phase A.

Results of experiment II. The average body weight, daily concentrate consumption, and hemoglobin level data for phase A of this study are presented in table 4.

Of the various treatment groups, a negative response was recorded in groups 1a, 2a, and 5a. Furthermore group 3a, which received vitamin B_{12} and cobalt injections, showed a

[9] To prepare the acid-soluble fraction of liver extract, the preparation was subjected to a 24 plate counter-current distribution using 42% phenol in *n*-butanol in one phase and 3% glacial acetic acid in H_2O (adjusted to pH 2.0) in the other phase. Both phases were equilibrated in order to prevent a change in volume. The peak of vitamin B_{12} activity is approximately at tube 7 at the left. The acid fraction was collected from tubes 19, 20, 21, 22 and 23 from this system and concentrated to a suitable volume to give 15 injectable U.S.P. units of the original material in each milliliter of the acid fraction.

[10] The vitamin B_{12} concentrate was prepared by treating Lederle (15 U.S.P. units) liver extract with glacial acetic acid so as to make a 3% acetic acid solution, which is then adjusted to pH 2.0 using HCl. This solution is then exposed to a 24 plate counter-current distribution using 3% glacial acetic acid in H_2O at pH 2.0 in one phase and 33% phenol in *n*-butanol in the other phase (each phase saturated with respect to the other). Tubes 10, 11, 12, 13, 14, 15, and 16 were combined, concentrated to a suitable volume and subjected to a second 24 plate counter-current distribution using the same conditions as above. Tubes 11 through 18 were then combined and concentrated to give 15 μg of vitamin B_{12} per milliliter of preparation.

TABLE 4

Summary of the body weights, feed consumption and hemoglobin levels obtained in experiment II, phase A

LOT NO.	1a	2a	3a	4a	5a	6a
TREATMENT	Cobalt-deficient	Pteroylglutamic acid injections	B_{12} and cobalt injections	Liver extract injections	Liver extract fed	Cobalt fed
No. of lambs	2	3	3	3	2	2
Ave. body weight, lb.						
Initial	58.4	53.8	45.8	53.7	51.0	43.7
1st week	60.6	56.1	51.5	58.9	51.0	50.8
2nd week	55.2	50.2	48.1	62.1	47.3	53.2
3rd week	53.5	49.9	50.3	64.7	47.6	56.2
4th week	51.7	47.8	50.8	66.9	44.2	59.1
Final	50.1	47.9	53.2	66.1	44.2	60.7
Ave. daily concentrates offered, lb.	1.08	1.08	1.08	1.08	1.08	1.08
Ave. daily concentrates consumed, lb.						
Pretreatment week	0.51	0.62	0.36	0.64	0.49	0.38
1st week	0.37	0.61	0.37	0.90	0.41	0.38
2nd week	0.28	0.52	0.50	1.06	0.48	0.95
3rd week	0.33	0.59	0.57	1.08	0.45	1.08
4th week	0.21	0.45	0.58	1.08	0.35	1.08
Final	0.12	0.30	0.51	1.08	0.19	1.08
Ave. hemoglobin level, gm/100 ml						
Initial	11.08	9.05	10.20	8.16	8.28	11.08
1st week	....	...	...	...	...	...
2nd week	10.71	8.07	9.16	8.28	8.20	9.14
3rd week	9.88	7.95	8.35	8.56	8.10	9.18
4th week	9.56	7.60	8.30	9.25	7.46	9.92
Final	8.80	6.81	7.51	9.50	7.18	9.55

marked decline in mean hemoglobin level and decreased concentrate consumption, although average body weight reflects a fair response. However, the response with respect to the latter criterion does not equal the beneficial effect noted in groups 4a and 6a. A striking response in body weight and concentrate intake was noted in lambs receiving injections of liver extract or oral dosages of cobalt, the average body weight increasing 12.4 and 17.0 lb., respectively, for the two groups during the test period. Both groups began consuming 100% of the concentrates offered during the third week of the experiment, and they continued at this level of consumption throughout the remainder of the study. Although the roughage consumption has not been presented, it followed a trend similar to that of concentrate intake. In groups 4a and 6a the response in hemoglobin level was not as impressive as the upward trend in body weight and feed intake. Liver extract injections resulted in a gradual and continuous rise in hemoglobin level; however, the cobalt-fed lambs showed a sharp drop followed by an increased level during the third week of the experiment. A beneficial influence was evident more quickly in group 4a than in group 6a.

Besides the differences in the above measurements, notes on the general appearance of the lambs during the course of the study also indicate curative effects in groups 4a and 6a and a lack of such response in the remaining groups. Increased thriftiness and agility followed within one week after the initiation of liver extract injections or oral cobalt administration, whereas the other lambs continued to show dullness, extreme weakness, and cachexia.

The results of phase B of the test are presented in table 5.

All lambs injected with the acid extract of the liver preparation continued to lose in body weight. The appetite continued to fluctuate at a low intake, which is particularly characteristic of cobalt-deficient animals. The mean hemoglobin level continued to diminish. It is noteworthy that one lamb in this group died of what was considered severe and typical cobalt deficiency during the second week of treatment. Similarly, a

negative response was obtained in lambs receiving injections of vitamin B_{12} concentrate, although the data reveal a very slight increase in body weight.

Group 3b, which received the liver extract injections, gained 10.6 lb. during the 4 weeks of the test. Improvement in appetite was apparent; however, this response was not as striking

TABLE 5

Summary of the body weights, feed consumption and hemoglobin levels obtained in experiment II, phase B

LOT NO.	1b	2b	3b
TREATMENT	Acid extract of liver extract	Vitamin B_{12} concentrate	Liver extract
No. of lambs	3 [1]	3	2
Ave. body weight, lb.			
Initial	44.8	50.2	43.6
1st week	43.1	52.1	49.4
2nd week	43.8	52.0	50.6
3rd week	41.2	51.7	52.3
4th week	...	...	54.2
Concentrates offered, lb.	1.08	1.08	1.08
Ave. daily concentrates consumed, lb.			
Pretreatment week	0.19	0.18	0.20
1st week	0.12	0.11	0.43
2nd week	0.07	0.17	0.51
3rd week	0.14	0.12	0.80
4th week	...	...	0.76
Ave. hemoglobin level, gm/100 ml			
Initial	7.39	7.38	7.20
1st week	6.63	7.08	7.16
2nd week	6.27	6.98	6.78
3rd week	5.71	6.63	6.98
4th week	...	...	7.44

[1] One lamb died during second week of the test.

as in the comparably treated groups in phase A. The mean hemoglobin level varied somewhat but, unlike the other groups in phase B, there was not a continued decrease in the level of this blood element. The lambs in group 3b showed a drop in hemoglobin level for the initial half of the study, after which

a slight increase was recorded. The general appearance of the lambs in this group indicated a positive response.

DISCUSSION

The results of these studies revealed that in cobalt deficiency the ability to digest crude fiber is apparently unaltered; at least when considering the entire alimentary tract. If crude fiber breakdown in the rumen is depressed by an inadequate cobalt intake, this is more than compensated for by digestion in the lower intestine. Actually, since the cobalt-deficient lambs were more efficient in digesting crude fiber than the supplemented lot, and since they consumed a ration containing approximately 50% more roughage, it seems unlikely that the capacity of the deficient lambs to digest crude fiber had been modified. On the other hand, the superior ability of deficient lambs to break down crude fiber is undoubtedly a reflection of the depressed feed intake, in accordance with the observations of Mitchell ('42).

The significance of the decreased capacity of deficient lambs to digest the ether-soluble and nitrogen-free extract fractions of the feed is an enigma. However, it does not seem that this depressed digestibility is of sufficient magnitude to be of major importance in cobalt deficiency.

Bowstead et al. ('42) first stated that the feeding of cobalt to the deficient animal must lead to a more efficient utilization of feed. From the data presented above it is apparent that any superior efficiency must occur in metabolism beyond the stage of digestion. This may not seem in accord with the observation of Gall et al. ('49), who noted a decreased microbial population in the rumen of deficient lambs. However, the cellulose-digesting organisms are apparently not affected; or, if so, they are not influenced sufficiently to cause a depressed digestibility at the feed intake levels recorded in this study.

The investigation of the therapeutic activity of some biological products adds support to the theory that cobalt deficiency is not a deficiency of cobalt per se but a lack of some other metabolite or metabolites whose production or activity is de-

pendent upon an adequate cobalt intake. The data presented indicate that liver in the form of liver extract does contain some factor or factors which are capable of curing the symptoms of cobalt deficiency. It is reasonable to assume that this activity cannot be attributed to the cobalt content of the extract, since even larger injections of cobalt in the form of various salts are therapeutically ineffective. Furthermore, the results agree in part with those of Filmer ('33), who noted that feeding raw or dried liver gave curative action although the ash of an equivalent amount of liver possessed no activity. His observations indicate that liver possesses corrective activity and that the source of the activity is organic in nature.

Unlike the results of Filmer, we did not obtain beneficial effects by oral administration of the liver preparation; however, varying amounts of the liver fed may resolve this disagreement. The absence of a response in deficient lambs fed, rather than injected, the liver extract may be the result of destruction of the activity or inefficient absorption from the alimentary tract.

A comparison of the rate of response of cobalt-deficient lambs to oral administration of cobalt and to subcutaneous injection of liver extract is noteworthy. Deficient lambs receiving cobalt orally continued to show a drop in mean hemoglobin level and in average daily concentrate intake during the initial week of therapy. Similar findings were noted earlier by Smith et al. ('50). Contrarily, lambs which received liver extract injections showed an immediate response, as indicated by all criteria. This difference suggests that the liver therapy is more directly supplying the deficient metabolite or metabolites than is orally administered cobalt. Perhaps a latent period is essential for the ruminal microorganisms to synthesize some vitamin-like material following stimulation by cobalt therapy.

Failure to note beneficial effects resulting from injection of pteroylglutamic acid, crystalline vitamin B_{12}, or vitamin B_{12} concentrated from a natural source eliminates these metabolic products as intermediaries in cobalt metabolism. The ineffec-

tiveness of the vitamin B_{12} products confirms the preliminary studies of Becker et al. ('49). Furthermore, the acid-soluble fraction of the liver preparation which was used in this study did not possess therapeutic activity. Further attempts to concentrate and perhaps identify the active factor or factors in the liver preparation are underway.

SUMMARY

The digestibility of a ration of shelled yellow corn, powdered whole milk, and cobalt-deficient hay was determined with 6 cobalt-deficient and 6 cobalt-fed lambs. The results reveal that the cobalt-fed lambs more efficiently digested the ether-soluble and nitrogen-free extract fractions of the ration. However, the deficient lambs showed a significantly greater coefficient of apparent digestibility for the fibrous fraction of the ration; an effect attributed to the depressed feed intake. Apparently, cobalt deficiency does not markedly affect the cellulose-splitting microorganisms of the rumen.

Subcutaneous injection of 10 mg of pteroylglutamic acid per lamb daily or the simultaneous injection of 1 μg of vitamin B_{12} and 1 mg of cobalt per lamb daily were therapeutically ineffective in cobalt deficiency in lambs. Cobalt feeding gave the usual beneficial response, whereas the negative controls continued to show a depressed appetite, body weight losses, and low hemoglobin levels. Subcutaneous injection of 15 U.S.P. injectable units of a purified liver extract daily per lamb was effective in the treatment of cobalt deficiency, as measured by all criteria; however, oral administration of the same dosage level was of no therapeutic value.

The results indicate that the liver preparation contained some factor or group of factors which possess curative activity for cobalt-deficient animals. This apparently is not cobalt. Preliminary efforts to concentrate the active fraction from the liver extract by means of counter-current distribution technics were unsuccessful.

ADDENDUM

Data more recently obtained and now ready for publication show that the amount of vitamin B_{12} injected in these lambs is suboptimum. Larger amounts of the vitamin do result in a positive response in cobalt-deficient lambs.

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EVALUATION OF SIX PARTIALLY PURIFIED PROTEINS BY RAT GROWTH, NITROGEN RETENTION BY YOUNG RATS AND LIVER REGENERATION

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TWO FIGURES

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The determination of the biological values of the 6 partially purified proteins examined in the cooperative study initiated by the Bureau of Biological Research of Rutgers University and studied by dog growth and dog maintenance in this laboratory yielded some unexpected results, due partially to the antitryptic content of the dried egg albumin (Mabee and Morgan, '51; Morgan et al., '51). It seemed of interest to compare these results with those obtained by somewhat similar procedures using rats.

EXPERIMENTAL

The growth experiment

Caged separately, weanling rats were fed a 24% casein diet until they weighed from 42 to 48 gm. At this time they were divided into 6 groups containing 10 animals (5 males, 5 females) each. The various litters were distributed evenly throughout the different groups. The diets fed to the 6 groups contained 10% protein from the following sources: casein, defatted whole egg powder, beef muscle powder, peanut flour, dried egg albumin, and gluten. These proteins were those

received from Rutgers University with the exception of the gluten, for which a sample was used which had a lower content of protein than the Rutgers gluten.

The diets were prepared by adding enough of the protein source to a non-protein basal diet to bring the protein level of the finished diet to 10%, 1.60% N. The basal diet had the following composition: Salts [1] 4%, cornstarch 76%, and fat [2] 20% (plus oil mix to provide 100 I. U. vitamin A, 10 units vitamin D, and 1 mg mixed tocopherols daily). Crystalline vitamins diluted with a small amount of the basal diet were added to the diets at 1% level so as to provide the following amounts in milligrams per cent: thiamine, riboflavin, and pyridoxine each 0.2, folic acid 0.2, calcium pantothenate and *p*-aminobenzoic acid each 1.0, inositol 25.0, biotin 0.02, choline 50.0, niacin amide 0.66.

In addition to the above mixture, 0.05 mg % of biotin was added to the egg albumin diet.

The animals were fed 50 gm of diet each week and if this amount was not sufficient they were given the non-protein basal diet ad libitum. An attempt was made to keep the total intake equal in all of the groups, but the appetite of the gluten group was so decreased that it did not seem wise to restrict the other groups to its lowered intake.

Two series of growth experiments were performed by this procedure.

The nitrogen retention experiment

At the termination of the 28-day growth period, a determination of the urinary and fecal nitrogen of the animals in the 6 groups was made. The collection was extended over a 4-day period and the collections from three animals in each group were pooled. Two collections were made from each animal during the period, and the urine and feces pooled separately so

[1] Hubbell, R. B., L. B. Mendel and A. J. Wakeman. J. Nutrition, *14:* 273, 1937.
[2] Hydrogenated vegetable fat.

that the sample represented the collection from three animals for 4 days. Three such collections were made for each group.

Since excellent growth and nitrogen retention were found in the rat experiments with egg albumin but poor nitrogen absorption in the case of dogs, it seemed desirable to determine the true digestibility of this protein when fed at a high level to rats. Six adult rats, 200 to 400 gm in weight, were placed on the non-protein basal diet to determine their endogenous nitrogen output, then on the 40% egg albumin diet (5.97% N), and these collection periods were repeated. A 4-day adjustment period was followed by 4 days of collection in each case.

The liver regeneration experiment

Three series of liver regeneration experiments were carried out, using the technique recommended by Harrison and Long ('45). In the first series mature rats one to two years old and of an average weight of 300 gm were used. In the second and third series rats three to 4 months old, weighing 200 to 250 gm. were used. The younger animals stored more liver nitrogen than the older group under the same circumstances.

The diets contained 12 or 18% protein, 14% fat in the first series and 25% in the other two series. The salt mixture, 2.5%, and crystalline vitamins as described in connection with the growth experiment, along with sucrose to make the diets isocaloric, completed the diets. The test animals were fasted for 48 hours and fed the diets for 4 days, and liver weights and nitrogen contents were determined.

RESULTS AND DISCUSSION

The growth experiments are summarized in table 1. The order of protein efficiency was the same in both experiments, with egg albumin receiving the highest rating, whole egg next, casein and beef muscle nearly alike and next, peanut flour and gluten following in that order. The gluten did little more than maintain the animals at this level. These values are in

the same order as those obtained by Ruegamer et al. ('50) by similar procedures with these proteins.

The nitrogen retention by the young rats (table 2) places these proteins again in the same order. The true digestibilities and biological values were not determined, since no endogenous excretion levels were obtained.

If the composition of the weight gains of these young rats is calculated from the growth data (table 1) and the retention

TABLE 1

Efficiency of 6 partially purified proteins for growth of rats

PROTEIN	SERIES	NO. OF RATS	INITIAL WEIGHT	GAIN IN 28 DAYS	TOTAL N INTAKE	GAIN PER GM EATEN	S. E. [1]
			gm	*gm*	*gm*	*gm*	
Egg albumin	1	12	47	59	3.2	18.4 ±	0.77
	2	10	44	54	3.3	16.3 ±	0.50
Whole egg	2	10	44	48	3.2	15.0 ±	0.47
Beef muscle	2	10	44	45	3.3	13.4 ±	0.60
Casein	1	13	47	45	3.2	14.0 ±	0.61
	2	10	44	41	3.2	12.8 ±	0.44
Peanut flour	1	12	47	27	3.1	8.6 ±	0.56
	2	10	44	29	3.3	8.8 ±	0.57
Gluten	1	13	47	11	2.8	3.9 ±	0.17
	2	10	44	6	2.7	2.4 ±	0.17

[1] Standard error of the mean gain per gram nitrogen eaten.

data (table 2), a fairly constant factor, 3.7 to 4.0% nitrogen, emerges (table 3). An exception is seen in the gluten group, which yields the value 11.7. This may be correlated with the share of maintenance involved in the metabolism of this static group or in the selective utilization of amino acids for production of non-tissue proteins (fig. 1).

The absorption of egg albumin ntrogen by adult rats (table 4) was nearly complete; 99% true digestibility as compared with 54% by adult dogs (Morgan et al., '51). If biological value can properly be calculated at such a luxus consumption level, it

was 92 for this group of animals. Both young and adult rats appeared able to denature completely the antitrypsin of egg white. Egg albumin studies based on rat feeding experiments may therefore be misleading in some respects. In particular, the ability of human infants and children to utilize this food remains to be established.

TABLE 2

Apparent absorption and retention of nitrogen by young rats from 6 partially purified proteins, in milligrams per rat per day

PROTEIN	INITIAL WT.	FOOD INTAKE	N INTAKE	FECAL N	URINE N	N ABSORBED	N RETAINED	APPARENT DIGESTIBILITY	N RETAINED PER GM ABSORBED
	gm	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*%*	*gm*
Whole egg	93	8.3	133	14	38	119	81	89	0.68
Egg albumin	98	7.5	124	9	39	115	76	93	0.66
Beef muscle	89	7.7	129	10	52	119	67	92	0.56
Casein	85	6.6	108	7	48	101	53	94	0.52
Peanut flour	72	7.5	126	14	70	112	42	89	0.38
Gluten	51	5.3	85	5	56	80	24	94	0.30

TABLE 3

Composition of gain by young rats fed 6 proteins

PROTEIN	N RETAINED PER GM EATEN	GAIN PER GM N EATEN	N CONTENT OF TISSUE ADDED
	gm	*gm*	*%*
Egg albumin	0.61	16.3	3.7
Whole egg	0.61	15.0	4.0
Beef muscle	0.52	13.4	3.8
Casein	0.49	12.8	3.8
Peanut flour	0.33	8.8	3.8
Gluten	0.28	2.4	11.7

The liver regeneration experiments dealt chiefly with the utilization of whole egg and egg yolk proteins. In the first experiment with large adult rats casein was found to provide significantly better regeneration than whole egg, and gluten about the same as whole egg (table 5). These values check very closely with those reported by Chow, Alper and deBiase ('48).

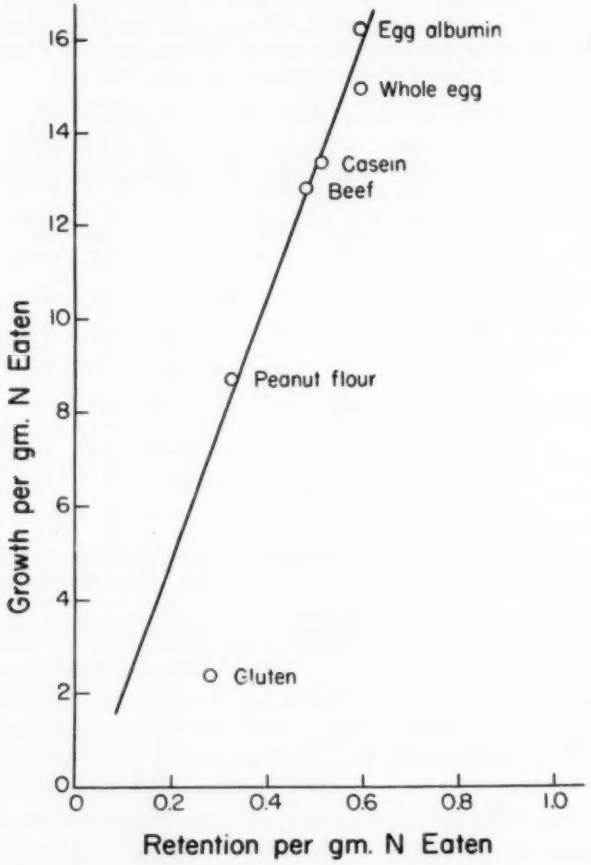

Fig. 1 Relationship of growth per gram N eaten and N retention per gram N eaten by young rats fed 6 proteins.

Heating the casein, defatted whole egg powder and gluten at 140°C. for 15 minutes in the dry state in each case reduced the liver regeneration values slightly. In the second experiment the defatted whole egg powder was compared with raw egg yolk and raw whole egg and found to be equivalent to the latter and superior to the former. The diet containing the 18% level

TABLE 4

Digestibility and biological value of egg albumin fed at 40% level to mature rats, gm N/rat/day

NO. OF RATS	BODY WT.	N INTAKE	FECAL N	METABOLIC FECAL N	URINE N	ENDOGENOUS URINE N	N ABSORBED	N RETAINED	TRUE DIGESTIBILITY	BIOLOGICAL VALUE
	gm	gm	gm	gm	gm	gm	gm	gm	%	%
6 ♀	214			0.013		0.216				
	219	2.63	0.035		0.418		2.61	2.40	99	92
7 ♀	206			0.006		0.210				
	222	2.45	0.034		0.460		2.42	2.17	98	90
8 ♀	216			0.015		0.242				
	225	2.27	0.022		0.380		2.26	2.12	100	94
1 ♂	390			0.019		0.346				
	398	3.70	0.044		0.669		3.67	3.35	99	91
11 ♂	374			0.017		0.388				
	373	3.65	0.041		0.625		3.63	3.39	99	94
3 ♂	410	4.18		0.018		0.350				
	421		0.061		0.770		4.14	3.72	99	90
	Average								99	92

of raw egg yolk protein produced less liver protein regeneration than did that containing 12% egg yolk protein. This led to the suspicion that some constituent of the raw egg yolk may have exerted an inhibiting action. The third experiment was designed to test the possibility that cholesterol might have this effect. Defatted dried whole egg was used to supply 12 and 18% protein in the diets, and for additional groups cholesterol

was added in the amounts present in the 12 and 18% egg yolk protein diets. These proved to be 1.4 and 2.1% cholesterol. The fat content of the livers of the rats fed these diets was not measured, but the liver total solids were very little changed by the 4 days' intake of these cholesterol-rich diets. The regeneration of liver protein was significantly less at each level in

TABLE 5

Influence of dietary protein upon regeneration of liver protein [1]

EXPERIMENT	PROTEIN LEVEL	GROUP AND DIET	LIVER NITROGEN, MG PER 100 GM BODY WT.	LIVER NITROGEN INCREMENT OVER FASTING LEVEL		LIVER SOLIDS
				Mg per 100 gm body wt.		
	%				%	%
1		Unfasted controls	95 ± 3.3 [2]	16	20	31.4
		Fasted controls	79 ± 2.8			34.7
	18	Raw whole egg	92 ± 3.3	13	16	32.3
	18	Dried defatted whole egg	94 ± 2.4	15	19	32.3
	18	Dried defatted whole egg heated	89 ± 3.5	10	12	31.9
	18	Gluten	95 ± 5.2	16	20	32.0
	18	Gluten heated	89 ± 5.1	10	12	32.2
	18	Casein	100 ± 1.6	21	26	33.0
	18	Casein heated	96 ± 3.7	17	21	32.6
2		Unfasted controls	149 ± 8.8	33	28	32.1
		Fasted controls	116 ± 3.9			30.1
	12	Defatted whole egg	142 ± 7.9	26	22	31.1
	18	Defatted whole egg	141 ± 5.1	25	21	31.4
	12	Raw egg yolk	132 ± 2.7	16	13	32.1
	18	Raw egg yolk	125 ± 5.7	9	8	31.7
	18	Raw whole egg	145 ± 3.0	29	25	33.1
3		Unfasted controls	118 ± 4.8	27	30	30.7
		Fasted controls	91 ± 3.3			33.0
	12	Defatted whole egg	118 ± 3.6	27	30	30.1
	12	Defatted whole egg plus 1.4% cholesterol	111 ± 3.0	20	23	30.8
	18	Defatted whole egg	132 ± 6.7	41	46	30.3
	18	Defatted whole egg plus 2.1% cholesterol	124 ± 2.4	33	37	32.3
	18	Defatted whole egg plus 2.1% cholesterol, plus 3.0% DL-methionine	107 ± 3.1	16	18	33.2

[1] Five or 6 rats in each group.
[2] Standard error of the mean.

the presence of cholesterol than in its absence. The addition of 3.0% DL-methionine to the cholesterol-containing 18% protein diet still further depressed the liver protein production.

If the values of these 6 proteins for dog growth, rat growth and dog maintenance be compared (fig. 2), it is evident that whole egg, beef and casein were alike in promotion of dog growth and that egg albumin was of little value to young dogs

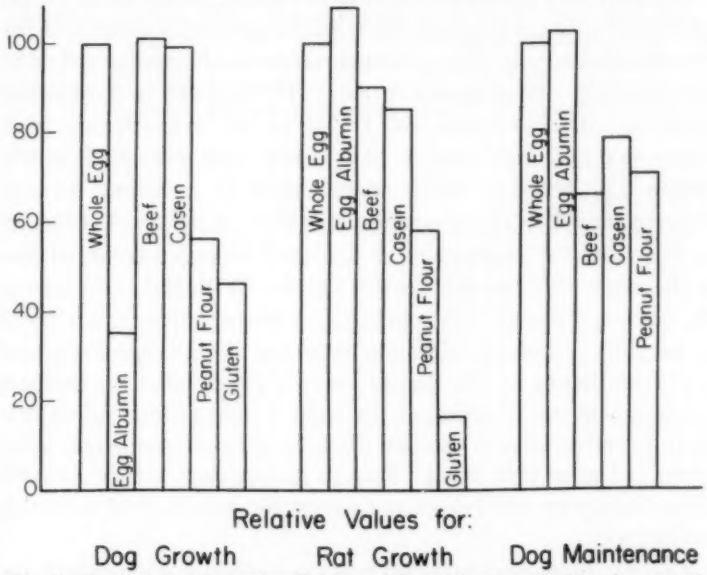

Fig. 2 Comparison of protein efficiency of 6 proteins measured by dog growth, rat growth and maintenance of N balance in adult dogs.

because of its indigestibility, but that both whole egg and egg albumin were superior to all the other proteins for rat growth. The values for maintenance of nitrogen balances in adult dogs were similar to those for growth in rats, except for beef, which was less valuable for this purpose than any of the other 4 proteins examined. Inadequacy of beef muscle proteins for production of hair protein may account for this discrepancy. The dogs used in these experiments were cocker spaniels with characteristically luxuriant pelage.

Species differences for both growth and maintenance are indicated by these comparisons and point to the need for parallel experiments with two or more species for estimation of both the nutritive values and the toxic properties of foods.

SUMMARY

Six partially purified proteins previously compared by the criteria of dog growth and dog maintenance were examined for growth efficiency in rats, nitrogen retention by young rats and liver protein regeneration in rats. The growth in grams per gram of nitrogen eaten was found to be: egg albumin 16.3, whole egg 15.0, beef muscle 13.4, casein 12.8, peanut flour 8.8, gluten 2.4. Similar values were found in a second growth experiment. The apparent digestibilities were much alike, 89 to 94%, and the proportion of absorbed nitrogen retained was in the same order as the growth values. With the exception of the gluten, a linear relationship was found between the gains in weight per gram of nitrogen eaten and the nitrogen retained per gram eaten by the young rats. This is taken to indicate constancy of composition of the body weight gains. Adult rats were found to digest the egg albumin almost completely even when fed at a 40% level. This is in contrast with only 54% digestibility by adult dogs, caused by the presence of an active antitrypsin.

Liver protein regeneration was most efficient with casein and gluten, slightly lower with whole egg and significantly retarded with egg yolk or with whole egg to which cholesterol was added in the amounts provided by the raw egg yolk. This decrease in liver regeneration in the presence of cholesterol was not accompanied by an increase in liver solids and therefore probably not by an increase in fat deposition.

The importance of recognizing species differences in the utilization of food proteins is indicated by these differences between the dog and the rat with respect to the absorption of egg albumin.

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STUDIES ON THE NATURE OF THE NUTRITIONAL DEFICIENCIES OF WHEAT GLUTEN MEAL [1]

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ONE FIGURE

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Several years ago it was demonstrated in this laboratory that the chick does not grow normally on a purified diet in which 18% casein is the only source of amino acids because such a diet supplies suboptimum levels of arginine, glycine and cystine or methionine. It was found that these deficiencies could be corrected by including in the diet 0.3% L-cystine and 10% gelatin, the latter as a source of arginine and glycine. Such a modified diet was found to sustain growth as rapid as that observed with a good practical poultry ration (Hegsted et al., '41a, b; Briggs et al., '42). Diets having this basic composition have subsequently been employed with much success in studies of the B vitamins.

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We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins and for the vitamin B_{12} concentrate; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Co., Inc., Chicago, Ill., for gelatin; and to E. I. DuPont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vitamin D_3.

Much of the preliminary work leading to the studies reported here was carried out by A. E. Harper and A. Lepp.

The general objectives of the work reported here were to investigate certain anomalies regarding the nutritive value for the chick of wheat gluten meal and to determine if purified casein meets growth requirements, in addition to furnishing simple amino acids, which are not supplied by wheat gluten protein. Our interest in wheat gluten arose from an earlier observation that 10% casein *or* 10% wheat gluten, or a combination of 5% of each, were equally effective as supplements to a low casein diet (12% casein, 10% gelatin and 0.3% methionine) in bringing about optimum growth of the chick. However, it was further noted that when all of the casein in such a diet was replaced by wheat gluten at a level of 22%, an extremely poor rate of growth resulted — somewhat less than one-third of optimum in a 4-week experimental period.[2] This was surprising, inasmuch as available amino acid analyses (see table 1) indicated that this diet supplied quantitatively all of the amino acid requirements of the chick with the exception of lysine, and that the deficiency of this amino acid was not severe. Furthermore, substantially higher levels of gluten were tested, such that even lysine was supplied in considerable excess, in the absolute sense, and the growth which resulted, although somewhat improved, fell far short of optimum. Lysine supplements were found to be only partially effective in improving the diet.

In the work reported here our procedure was to determine if amino acid supplements would be effective in bringing about maximum growth on the 22% gluten diet, to determine what additional amino acids would be required, both qualitatively and quantitatively, and to attempt to ascertain the reasons for such additional requirements.

EXPERIMENTAL

Straight-run, crossbred (New Hampshire ♂ ♂ × Single Comb White Leghorn ♀ ♀) chicks were employed in these experiments. They were the progeny of hens fed the previously

[2] Unpublished results obtained in this laboratory by A. E. Harper and A. Lepp.

described diet B-1 (Robblee et al., '48). The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum in all experiments.

TABLE 1

Approximate percentage composition of essential amino acids in the unsupplemented gluten and casein rations, as calculated from the literature [1]

AMINO ACID	DIET			
	(1) 18% Casein, 10% gelatin	(2) 22% Gluten, 10% gelatin	(3) 32% Gluten, 10% gelatin	(4) Requirement
	%	%	%	%
Arginine	1.5	1.4	1.7	1.2 [2]
Histidine	0.53	0.44	0.62	0.3 [2]
Tryptophan	0.18	0.18	0.26	0.18 [2]
Methionine	0.6	0.6	0.8	0.5 [2]
Phenylalanine	1.0	1.2	1.4	0.9 [2]
Threonine	0.73	0.58	0.67	0.45 [4]–0.6 [2]
Lysine	1.65	0.72	0.85	0.9 [2]
Leucine	1.8	1.1 (2.0)	1.6	1.4 [2]
Isoleucine	1.0	0.7 (0.8)	1.1	0.6 [2]
Valine	1.2	0.7 (0.8–1.0)	0.9	0.8 [2]
Glycine	2.7	3.3	3.5	1.0 [5]

[1] The values were calculated, for the most part, from the data of Block and Bolling ('40, 1945 printing). However, the values for leucine, isoleucine, and valine were calculated on the basis of a microbiological assay of wheat gluten performed in this laboratory by Dr. Olaf Braeken, the latter values being substantially lower than those calculated from Block and Bolling, given in parentheses. However, the chick growth results reported here tend to confirm the higher values, for none of the amino acids in question was found to be deficient in the 22% gluten-10% gelatin ration.

[2] Almquist ('47).

[3] Wilkening et al. ('47).

[4] Grau ('49).

[5] Almquist ('45).

The purified positive control diet contained the following: alcohol-extracted casein 18 gm, gelatin 10 gm, dextrin 60 gm, salts V (Briggs et al., '43) 6.0 gm, soybean oil 5 gm, L-cystine 0.3 gm, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, pyridoxine hydrochloride 0.4 mg, nicotinic acid 5.0 mg, calcium pantothenate 2.0 mg, biotin 0.055 mg, folic acid 0.05 mg, *p*-am-

inobenzoic acid 10 mg, inositol 100 mg, choline chloride 150 mg, menadione 0.05 mg, α-tocopherol 0.3 mg, vitamin B_{12} 3 μg, and fortified halibut liver oil (60,000 U.S.P units of vitamin A, 6,000 A.O.A.C. units of vitamin D_3 per gram) 40 mg.[3] In the earlier experiments the halibut liver oil was mixed directly into the ration; however, this practice appeared to be correlated with a high incidence of nutritional encephalomalacia, presumably due to destruction of vitamin E in the prepared diets (Dam, '44). Therefore, in the later experiments this supplement was supplied by dropper in amounts correlated with the consumption of the diet and no further difficulties of this nature were encountered. In experiments 1 through 9 the vitamin B_{12} was supplied as the charcoal-soybean flour adsorbate[4] and in the subsequent experiments an aqueous solution of the crystalline vitamin was incorporated into the diet. In the negative control diet all of the casein (except for slightly less than 100 mg used as a base for introducing the various B vitamins) and 4 gm of the dextrin were replaced by 22 gm of wheat gluten meal.[5] The various amino acid supplements were added at the expense of dextrin on a gram for gram basis.

The approximate amino acid composition of the two gluten-gelatin basal diets employed and of the positive control diet, as well as the quantitative amino acid requirements which have been tentatively established for the growing chick, have been summarized in table 1. Kjeldahl nitrogen values were: gluten 12.7%, gelatin 16.1%, and casein 13.1%.

In several of the experiments, amino acid supplements were added to the gluten-gelatin diet in such amounts that the level of the particular amino acid in question was slightly higher than that which it enjoyed in the positive control diet. These supplements were calculated on the basis of the differences between the levels of the various essential amino acids in

[3] Abbott Laboratories' "Haliver Oil," fortified with crystalline vitamin D_3 to supply the activity indicated.

[4] Merck.

[5] From the Pure Gluten Food Co., New York, N. Y.

the two diets. In all cases where synthetic amino acids had to be used, the unnatural isomers were not considered to be part of the supplement and, therefore, twice the calculated level was employed; methionine, both isomers of which are known to be utilized by the chick, was the only exception to this rule. Arginine and lysine were supplied as the monohydrochlorides, histidine as the monohydrochloride monohydrate.

It will be noted that most of the experiments were differential in character, being of short duration and usually involving an assay period of only 6 days, a procedure which was mandatory because of the high cost of certain of the amino acids used. These short-term assays involved chicks which were reared on a pretest diet for several days after hatching (a period which varied from 10 days to 4 weeks) and, at the proper time in each case, were carefully divided into the respective test groups on the basis of weight and percentage gain during the preceding three to 6 days. In order to obtain comparable experimental groups it was always necessary that several atypical birds be discarded at this stage. Although the various test diets were given immediately upon division of the birds, the initial recording of weights and food consumption was postponed for approximately 24 hours. Weights were usually recorded daily, although this was done on alternate days in experiments 1 and 6 (appropriate corrections were employed in calculating the rates of gain in the latter two cases). For the sake of uniformity and in order that the results might be more directly compared with those to be found in the literature, the growth responses of the individual birds were calculated as average per cent gain per day (as defined by Grau and Almquist, '43), and an average was taken for the groups.

In most of the experiments we have also reported the grams gained by each group of animals per gram of diet consumed. The maximum differences in calculated results which we have noted between duplicate groups within the same experiment are 0.8% per day and 0.01 gm gained per

gram consumed, respectively. The latter criterion, since it has yielded the more readily reproducible results under the conditions of these expermients, has been given the greatest weight in the interpretation of the results; however, it was necessary that both criteria be considered, since it was possible to increase dietary efficiency substantially beyond that which was correlated with maximum rate of gain.

The pretest diet ordinarily employed was that of the positive control groups (in the experiments where crystalline vitamin B_{12} was used, the pretest level of this vitamin was usually 1 μg % instead of the experimental level of 3 μg %), although the negative control diet was used for this purpose in experiments 3 and 4.

The negative control diet was not used more extensively during the pretest period primarily because it was very frequently associated with the onset, as the birds reached approximately two weeks of age, of a syndrome very strongly suggestive of encephalomalacia and, to a lesser extent, of polyneuritis (head retraction) associated with a rapid decline in weight and certain death within approximately three days. The onset of this syndrome could not be prevented by carefully protecting the dietary source of vitamin E, or by oral supplementation with α-tocopherol. We have subsequently observed that the syndrome can be completely cured within approximately 12 hours by the subcutaneous injection of a massive dose of thiamine. This observation suggested the possibilities that the chick may have a higher requirement for this vitamin on a diet characterized by an amino acid imbalance or that, in reducing its food intake on such an unsatisfactory diet as is involved here, it is depriving itself of an adequate supply of the vitamin. The level supplied is fully adequate, not to say excessive, for chicks given a ration which otherwise promotes normal growth. The further possibility that the destruction of thiamine in the prepared diet is accelerated in the presence of commercial wheat gluten cannot as yet be discounted.

RESULTS

The results of the differential growth assays are summarized in table 2, and those obtained with the somewhat longer assays involving day-old chicks will be found in table 3 and figure 1. The experiments are numbered in chronological order. Since it became apparent as the work progressed that the ages and average weights of the birds at the beginning of the various experiments had a very substantial bearing upon the results, these variables have also been included in the tables.

Experiment 1 primarily involved supplements consisting of various combinations of the full percentage requirements of the essential amino acids as tentatively stated by Almquist ('47) — the supplement in diet 2 was only half the stated lysine requirement — and also the supplements calculated to be adequate to raise the levels of all essential amino acids in the 22% gluten ration to levels very slightly higher than the corresponding levels in the positive control ration (diet 9). The performance of group 9 clearly demonstrates that the latter supplements are more than adequate; indeed, this diet and, to an even greater extent, that of group 7 (to which the full minimum quantitative requirements of 10 essential amino acids were added) seem to be superior to the control diet (group 10). The responses of groups 2 and 3 show that a supplement of 0.45% L-lysine is more than adequate; that of group 5, as well as that of group 8 relative to group 6, clearly demonstrate a requirement for added threonine. The fact that the response of group 4 was not greater than that of group 3 did not, of itself, rule out a requirement for added tryptophan, although the extremely low level of this supplement in diet 9 — with which an optimum response was obtained — tended to suggest that such an additional requirement did not exist.

The results in experiment 2, a stepwise breakdown of the supplements used in diet 9, confirm the adequacy of the supplements used in that diet and tentatively show that the sup-

TABLE 2

Effect of essential amino acid supplements on the nutritive value of gluten-gelatin rations for chick (experiments with groups of chicks pre-selected for weight and vigor)

EXPT. NO.	INITIAL AGE AND AVE. WT.	DIET AND GROUP NO.	DIET [1]	AVE. GAIN PER DAY	GAIN/FEED
				%	*gm/gm*
1	13 days	1	22% Gluten basal	1.8	0.23
	118 gm	2	22% Gluten + 0.45% L-lysine	4.9	0.45
		3	22% Gluten + 0.9% L-lysine	4.1	0.37
		4	Diet 3 + 0.5% DL-tryptophan	4.1	0.39
		5	Diet 4 + 0.6% DL-threonine	6.8	0.59
		6	Diet 4 + 0.6% DL-methionine 1.8% DL-phenylalanine 1.6% DL-valine	3.4	0.29
		7	Diet 6 + 0.6% DL-threonine 1.2% DL-isoleucine 1.4% L-leucine 1.2% L-arginine 0.3% L-histidine	8.3	0.75
	(10 chicks per group)	8	Diet 5 + 0.6% DL-methionine 1.8% DL-phenylalanine 1.6% DL-valine	6.9	0.58
		9	22% Gluten + 1.0% L-lysine 0.6% DL-threonine 0.08% DL-tryptophan 0.1% L-arginine 0.1% L-histidine 0.2% DL-methionine 0.7% L-leucine 1.2% DL-isoleucine 1.4% DL-valine	7.9	0.70
		10	18% Casein (control)	7.5	...
2	11 days	11	22% Gluten basal	2.2	0.26
	93 gm	12	22% Gluten + 1.0% L-lysine 0.6% DL-threonine	4.5	0.47
		13	Diet 12+ 0.1 L-histidine	4.4	0.45
		14	Diet 13 + 0.7% L-leucine 1.2% DL-isoleucine 0.1% L-arginine	3.5	0.49
	(10 chicks per group)	15	Diet 14 + 0.2 DL-methionine 1.4% DL-valine 0.08% DL-tryptophan	6.7	0.58
		16	18% Casein (control)	7.0	0.55

TABLE 2 *(continued)*

EXPT. NO.	INITIAL AGE AND AVE. WT.	DIET AND GROUP NO.	DIET [1]	AVE. GAIN PER DAY	GAIN/FEED
				%	gm/gm
3	14 days	17	32% Gluten	2.7	0.30
	60 gm	18A	32% Gluten + 0.85% L-lysine	8.3	0.633
		18B	Identical with 18A	7.6	0.635
		19	22% Gluten +	4.5	0.43
	(10 chicks		1.0% L-lysine		
	per group)		0.6% DL-threonine		
			1.4% DL-valine		
		20	18% Casein (control)	11.4	0.72
4	17 days	21	22% Gluten +	6.2	0.46
	60 gm		0.4% L-lysine		
			0.2% DL-threonine		
			0.3% DL-valine		
	(4 chicks	22	Identical with diet 19	5.4	0.42
	per group)	23	Diet 22 + 0.08% DL-tryptophan	9.1	0.66
		24	Diet 23 + 0.7% L-leucine	7.8	0.49
5	30 days	25	22% Gluten + 0.4% L-lysine	5.3	0.46
	204 gm	26	Diet 25 + 0.2% DL-threonine	6.1	0.53
		27	Diet 26 + 0.3% DL-valine	4.8	0.46
		28	Diet 27 + 0.08% DL-tryptophan	6.9	0.59
	(4 chicks	29	Diet 28 + 0.2% L-arginine	6.8	0.56
	per group	30	Diet 29 + 0.2% L-histidine	6.0	0.56
		31	18% Casein (control)	6.5	0.61
6	17 days	32	22% Gluten basal	2.2	0.24
	135 gm	33	22% Gluten + 0.4% L-lysine	3.7	0.40
		34	Diet 33 + 0.2% DL-threonine	5.7	0.59
		35	Diet 34 + 0.08% DL-tryptophan	6.1	0.59
		36	Diet 34 + 0.3% DL-valine	5.7	0.53
	(10 chicks	37	Diet 36 + 0.08% DL-tryptophan	6.5	0.59
	per group)	38	Diet 36 + 0.04% L-tryptophan	6.4	0.57
		39	Diet 36 + 0.02% L-tryptophan	6.5	0.59
		40A	18% Casein (control)	7.2	0.65
		40B	Identical with 41A	8.0	0.65
8	16 days	46	22% Gluten +	5.8 (6.1)[2]	0.50
	139 gm		1.0% L-lysine		
			0.6% DL-threonine		
		47	Diet 46 + 0.08% DL-tryptophan	6.2 (7.0)[2]	0.53
		48	Diet 47 + 1.4% DL-valine	5.4	0.47
		49	Diet 46 + 1.4% DL-valine	5.4	0.42
		50	22% Gluten +	6.2	0.48
	(8 chicks		0.4% L-lysine		
	per group)		0.2% DL-threonine		
			0.3% DL-valine		
			0.08% DL-tryptophan		
			(Identical with diet 37)		
		51	18% Casein (control)	7.8 (7.2)[2]	0.59

TABLE 2 *(continued)*

EXPT. NO.	INITIAL AGE AND AVE. WT.	DIET AND GROUP NO.	DIET [1]	AVE. GAIN PER DAY	GAIN/FEED
				%	*gm/gm*
9	28 days 341 gm (3 chicks per group)	52	Diets 37 and 50	4.2	0.51
		53	18% Casein (control)	4.2	0.51
11	21 days 160 gm	56	22% Gluten + 1.0% L-lysine 0.6% DL-threonine	6.7	0.53
		57	Diet 56 + 0.2% DL-methionine	7.5	0.46
		58	Diet 57 + 0.08% DL-tryptophan	7.8	0.59
	(7 chicks per group)	59	Diet 58 + 0.1% L-arginine 0.1% L-histidine	7.2	0.58
		60A	18% Casein (control)	8.0	0.59
		60B	Identical with diet 60A	7.9	0.60
12	30 days 304 gm (7 chicks per group)	61	22% Gluten + 1.0% L-lysine 0.6% DL-threonine	5.0	0.60
		62	18% Casein (control)	5.4	0.61
13	19 days 181 gm	63	22% Gluten + 1.0% L-lysine 0.6% DL-threonine	6.3	0.57
		64	Diet 63 + 0.08% DL-tryptophan	6.5	0.60
		65	Diet 64 + 0.2% DL-methionine	7.3	0.62
	(8 chicks per group)	66	Diet 65 + 1.4% DL-valine	7.3	0.65
		67	Diet 66 + 1.2% DL-isoleucine	7.4	0.68
		68A	18% Casein (control)	7.5	0.61
		68B	Identical with 68A	7.2	0.60
14	26 days 274 gm	69	22% Gluten + 0.4% L-lysine 0.2% DL-threonine	5.09	0.584
	(8 chicks per group)		0.3% DL-valine 0.08% DL-tryptophan		
		70	18% Casein (control)	5.08	0.592

[1] All diets contained 10% gelatin and 0.3% L-cystine.

[2] The values for average per cent gain per day which are in parentheses were calculated on the basis of a 10-day growth period, whereas the values not in parentheses refer to a 6-day period (experiment 8).

plemental amino acids required in addition to lysine and threonine are among the three added in diet 15 — namely, methionine, tryptophan, and valine — although, as in the case of tryptophan in experiment 1, the supplements added in diets 13, 14, and 15 cannot be positively ruled out. The adequacy of the supplements used in diet 9 over a more extended period of time, beginning with the day-old chick, is clearly demonstrated by experiment 10, which was of 20 days' duration.

TABLE 3

Effect of essential amino acid supplements on the nutritive value of gluten-gelatin rations for the chick (experiments with groups of randomly chosen, day-old chicks; see also fig. 1)

EXPT. NO.	DURATION OF EXPT.	DIET AND GROUP NO.	NO. OF BIRDS	DIET [1]	FINAL WT.	GAIN/FEED
					gm	*gm/gm*
10	20 days	54	18	22% Gluten + 9 amino acids as in diet 9	189 ± 7.0	0.68
		55	18	18% Casein (control)	193 ± 5.7	0.59
15	11 days	71	19	22% Gluten + 1.0% L-lysine 0.6% DL-threonine 0.2% DL-methionine 0.08% DL-tryptophan	112 ± 3.9	0.85
		72	19	Diet 71 + 1.4% DL-valine	107 ± 3.5	0.85
		73	20	18% Casein	102 ± 2.9	0.70

[1] All rations contained 10% gelatin and 0.3% L-cystine.

In experiment 3 the response of group 19 demonstrated clearly that the next limiting amino acid after lysine and threonine is not valine. Furthermore, the response of group 18 — involving the 32% level of gluten supplemented with lysine — to a ration in which the levels of all essential amino acids were clearly more than adequate in the absolute sense (see table 1) makes it quite clear that something more than an absolute dietary deficiency of any essential amino acid was involved.

Experiments 4 and 5, involving rather small groups of birds, were preliminary in character. Experiment 4, in which the animals were essentially the same in size and age as those used in experiment 3, demonstrated quite clearly that the

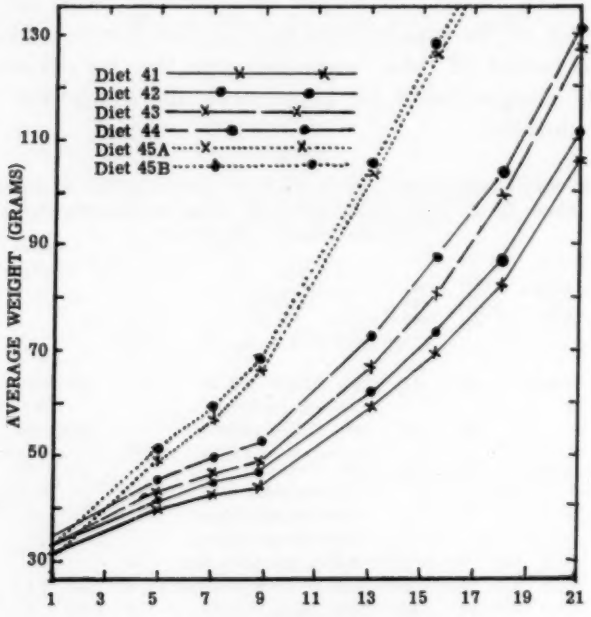

Fig. 1 (Experiment 7) Growth of day-old chicks on purified diets containing wheat gluten and gelatin supplemented with amino acids. Supplements were: for diet 41, 0.4% L-lysine + 0.2% DL-threonine; for diet 42, diet 41 + 0.3% DL-valine; for diet 43, diet 41 + 0.08% DL-tryptophan + 0.3% DL-valine (the latter added after one week); for diet 44, diet 42 + 0.02% DL-tryptophan. Diets 45A and 45B were duplicate positive controls. Each group contained 18 chicks.

next limiting amino acid after lysine and threonine is tryptophan and that additional leucine is not required. The good response of group 21 foreshadowed the adequacy of lower levels of lysine, threonine, and valine in further investigations. Experiment 5 involved the birds from groups 18 and

20 of experiment 3, which were subsequently reared for several days on the positive control diet prior to being divided again. The response of group 28 was interpreted as indicating that the relatively low levels of the supplemental amino acids in diet 28 would be completely adequate. The very marked activity of such a small amount of L-tryptophan as was added to this diet (compare with diet 27) was especially interesting. The responses of groups 29 and 30 indicated that additional arginine and histidine are not required.

Experiment 6 was projected as a verification and extension of the responses of groups 25 to 28 of experiment 5. However, the responses of groups 32 through 37 seemed to contradict these earlier results, for the difference between group 37 and the positive controls (groups 40 A and B) was rather large. Moreover, although tryptophan does show some activity in overcoming the inhibition of growth brought about by the addition of valine over and above lysine and threonine (as it did in the previous experiment, groups 27 and 28), its net enhancement of the diet supplemented with lysine and threonine alone is not appreciable (groups 34 and 35). However, an interesting observation in connection with the response of groups 36 through 39 is that as little as 0.02% L-tryptophan appears to be fully as active as 0.08% DL-tryptophan in overcoming this inhibition of growth due to supplemental valine.

A clue to the reason for the apparently contradictory results obtained was suggested by the outcome of experiment 7, a repetition of the more anomalous parts of experiment 6, employing day-old chicks. The results are presented as growth curves in figure 1. It can be clearly seen that the growth of the groups receiving the various supplemented gluten rations tended to be quite inferior for approximately the first two and one-half weeks, but that after that time the growth of these groups became considerably more rapid; indeed, the response of group 43 (whose diet was similar to diets 28 and 37, respectively, of experiments 5 and 6) eventually became fully as rapid as that of the control groups at

the corresponding weight level. The obvious implication, therefore, is that certain of the amino acid requirements of an older, larger bird are less, on a percentage basis, than those of a younger, smaller bird, even though both may be growing at a rapid rate. This concept has been verified by subsequent experiments, in which it has been demonstrated that, in addition to supplements of lysine, threonine, and tryptophan, the younger chick (under approximately 200 gm in weight) also requires for optimum growth a supplement of DL-methionine which is not required by the older chick. Thus, in experiment 5, the supplements added to diet 28 were entirely adequate to promote the optimum growth of birds 30 days of age and weighing 200 gm, on the average. These results have been fully confirmed with still larger birds in experiments 9 and 14. On the other hand, the same supplements failed to sustain this level of growth as diet 37 of experiment 6, where the birds initially weighed on the average only 135 gm. This result has been confirmed by the response of group 50 of experiment 8.

Experiment 8 was designed to determine if the difficulty, the general cause of which was suggested by the outcome of experiment 7, was due specifically to an inadequate supplement of one of the 4 amino acids already under investigation — namely, lysine, threonine, valine, or tryptophan — or to an additional requirement of the very young chick not met by any of these amino acids. Hence, higher levels of the various supplements were employed in diets 46 through 49. The results made it quite apparent that an additional amino acid was required. However, the fact that the growth responses of groups 46 and 47 relative to the control group (group 51) were markedly better after 10 days than after 6 days on experiment is a clear indication that this additional requirement drops off markedly as the birds approach the 200-gm weight level.

The results of experiments 11 and 13 demonstrate clearly enough that the additional supplemental amino acid required by the very young chick is methionine. Experiment 15 dem-

onstrates that our negative control basal ration will support the optimum growth of the day-old chick and superior diet utilization when supplemented with the amino acids lysine, threonine, tryptophan, and methionine. The response of group 72 makes it quite clear that supplemental valine is neither required nor of any particular benefit. Its use as a supplement was of considerable help in the course of the work, in bringing out the borderline character of the tryptophan level in the basal diet and thus establishing a requirement for a small supplement of this amino acid.

The results of experiment 12, though not entirely conclusive, suggest that even this small supplement of tryptophan is no longer required for optimum growth after the chick becomes sufficiently large (about 300 gm).

DISCUSSION

As a point of departure in explaining the reasons for the supplemental amino acid requirements of the chick on our 22% gluten basal ration, we can best consider the case of the chick weighing 200 gm or over. Here it was established that the only additional amino acids required to bring the growth response from a very poor one to an optimum one were quite small amounts of lysine (only slightly more than the amount necessary to overcome the absolute deficiency indicated in table 1), threonine, and tryptophan. Examination of table 1 shows that these are the three most limiting essential amino acids (if we accept the values for leucine, isoleucine, and valine given in parentheses in column 2, as the experimental results suggest we should). Since correcting the lysine deficiency did not result in anything approaching optimum growth, it is reasonable to conclude that the absolute requirements for these three amino acids are somewhat higher on our type of ration. This conclusion can be interpreted as an extension of the work only recently reported by Almquist ('49) and Almquist and Merritt ('50), which seems to demonstrate the dependence of the arginine and methionine requirements of the chick upon the over-all level of protein

in the diet (a similar observation regarding the lysine requirement was reported earlier by Grau, '48). This interpretation becomes plausible when we consider that the percentage requirements listed in table 1 were for the most part determined on a ration containing approximately 20% protein, whereas our diet contained approximately 28% protein. Of course these observations are actually a further confirmation of the well-established proposition that all the essential amino acids in the diet must be present in a proper ratio one to another, according to the relative requirements of the animal for each. Apparently, all the other amino acids were present in our rations in sufficient excess to bring about a deficiency of the three amino acids in question.

Although the results presented do not require us to postulate that inadequate digestion or varying rates of enzymatic release of certain essential amino acids — as suggested by the results of recent studies (Geiger and Hagerty, '49; Kligler and Krehl, '50) of the utilization of zein by the rat — are significant factors in the poor utilization of wheat gluten protein, these factors cannot be ruled out as yet. To do so would require a much more precise knowledge of the amino acid composition of wheat gluten than it is possible for us to obtain at the present time.

In this connection, some question might arise from the fact that the levels of tryptophan and methionine appear, on the basis of the calculations presented in table 2, to be approximately the same in both the positive control diet and the 22% gluten diet. This would suggest that perhaps the rates of enzymatic release of these two amino acids from wheat gluten were not sufficiently rapid to promote their full utilization by the animal. However, certain reports which have appeared since 1945, as well as certain as yet unpublished data obtained in this laboratory, suggest that the level of tryptophan in casein is substantially higher and that of methionine in gluten is somewhat lower than the values used in the preparation of table 1 (these values were: tryptophan

in casein — 1.2 gm, and methionine in gluten — 3 gm, respectively, per 16 gm nitrogen).

Concerning the apparent diminution of the methionine requirement relative to the requirements for the other amino acids as the chick increases in weight, only the most tentative sort of explanation can be offered. The possibility exists that this phenomenon is simply a manifestation of a generally higher requirement for the sulfur amino acids in the period immediately after hatching, during which the development of feathering plays a greater role in the over-all development of the animal than during later stages of growth.

SUMMARY

The ability of the chick to utilize the amino acids in wheat gluten has been shown to be correlated with the essential amino acid content of this material. A slight imbalance can be corrected by means of small amounts of supplemental amino acids (lysine, threonine, and tryptophan) in order to bring about optimum growth.

In the course of this investigation it has been demonstrated that the very young chick has a higher growth requirement for methionine relative to the other essential amino acids than does the somewhat larger, older chick.

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THE EFFECT OF SOME COMBINATIONS OF HUMAN FOODS ON THE GROWTH AND HEALTH OF THE LABORATORY RAT [1]

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The experiments reported here were originally undertaken as two independent investigations to estimate the nutritive quality of some food combinations which contained: (1) large proportions of cereal; and (2) egg at different levels. The criteria to be used in comparing the adequacy of these diets were the development, health, and reproductive performance of laboratory rats fed the various diets ad libitum from the time of weaning throughout life. However, study of the experimental data showed that a fruitful comparison could be made between the two series of diets as well as among the diets within each series; the present discussion is from the former point of view.

EXPERIMENTAL

Description of diets

Each of 8 major food groups was represented in the experimental diets by one or two typical foods which, in order to simulate human practices more closely, were cooked by accepted culinary methods. A concession was made to the eating

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habits of the laboratory rat by drying the cooked foods before combining them in the dietary mixture.

Diets 1 and 2 were characterized by a high content of cereal; white, whole-grain corn meal and polished rice, respectively, supplied 65% of the total calories of these two food combinations. Diets 3, 4, and 5 included considerably less cereal, more milk, meat, vegetables, and fat, and variable proportions of egg, ranging from sufficient egg to give 17% of the total caloric value in diet 3 to no egg at all in diet 5.

The proportions of the total calories contributed by the individual foods are summarized in table 1; relevant data from food supply and consumption surveys for the United States (U. S. Bureau of Human Nutrition and Home Economics, '48; U. S. Bureau of Agricultural Economics, '49) are also included for purposes of comparison.

Preparation of diets

All of the foods were cooked as for human consumption. The cooking procedures employed, which were standardized and duplicated at each repetition as closely as possible, were as follows:

Bread. Unenriched patent wheat flour was secured from the same mill twice during the experimental period and stored at 4°C. The bread was baked according to a commercial formula in common use; fat was omitted and a vitamin enrichment mixture [3] added in an amount to conform with the standard for enriched bread.

Corn meal and rice. White, whole-ground corn meal was secured from the same mill twice, and polished rice of the Blue Rose variety once, during the experiment and stored at 4°C. Both cereals were cooked in the same way; they were added to boiling distilled water in constant proportions of cereal to water and cooking was continued for one hour in a covered pan over boiling water.

[3] Type 4 "N-Richment-A," furnished through the courtesy of the Novadel-Agene Corporation, Belleville, New Jersey.

TABLE 1

Percentage of total calories contributed to experimental diets by individual foods, and comparison with U. S. food consumption and supply figures for major food groups represented

FOOD GROUPS REPRESENTATIVE FOODS	DIET					FOOD PER CAPITA, 1948:		
						Urban consumption, by yearly income [1]		Total average supply [2]
	1	2	3	4	5	Under $1,000	$7,500 and over	
Grain products:						28.7	21.4	24.0
Bread, white enriched	...	...	34.4	36.2	36.7			
Corn meal, white, whole grain	65.0	...	...	...	...			
Rice, polished	...	64.9	...	...	...			
Eggs:						2.5	2.9	2.6
Eggs, whole, dehydrated	1.8	1.7	17.3	2.6	0			
Meat, poultry, fish:						12.0	16.0	12.4
Beef, round	3.9	3.8	6.8	7.2	7.3			
Pork, loin	2.0	2.0	3.5	3.7	3.7			
Beans, peas, and nuts:						2.8	2.1	2.7
Beans, navy, dried	4.0	4.0	3.5	3.7	3.7			
Potatoes, sweet potatoes:						3.1	2.9	3.3
Potatoes, white	4.0	3.9	5.6	5.9	6.0			
Vegetables and fruits, all other:						7.0	10.2	6.3
Carrots	3.0	3.0	5.4	5.7	5.8			
Kale	2.0	2.0	2.5	2.7	2.7			
Dairy products other than butter:						10.9	17.5	13.8
Milk, whole, dried	9.9	9.9	16.0	16.8	16.9			
Fats and oils:						21.8	16.8	19.3
Hydrogenated vegetable oil	4.9	4.9	5.0	15.5	17.4			
Sugars, sweets: [3]								

[1] U. S. Bureau of Human Nutrition and Home Economics ('48).

[2] U. S. Bureau of Agricultural Economics ('49).

[3] Sugar, as such, was not included in the experimental diets; the yearly urban consumption at two income levels, and yearly supply of this commodity per capita were 11.2, 10.2, and 15.6% of total calories, respectively, in 1948 (U. S. Bureau of Human Nutrition and Home Economics, '48).

Eggs. Spray-dried whole egg [4] was reconstituted by allowing it to stand for 15 minutes with distilled water, and cooked over boiling water for 20 minutes.

Navy beans. The beans were washed in tap water, rinsed in distilled water, and simmered in distilled water over a gas burner for two hours. The partially cooked beans were then placed in a large pan and baked 4 hours at 122°C., water being added as needed.

Beef and pork. Beef rounds and pork loins were secured as needed, divided into pieces of convenient size and roasted in a Dispatch oven, the beef at an oven temperature of 163° to an interior temperature of 80°C., and the pork at an oven temperature of 176° to an interior temperature of 85°C.

Potatoes. Potatoes were secured in one lot and stored at 4°C., 85% humidity. They were boiled in the skins, which were removed before use.

Carrots. Carrots were procured by the bushel, as needed. They were California-grown and largely of the Imperator [5] variety. The carrots were topped, washed in tap water, rinsed in distilled water, pared with a vegetable scraper and cut into one-quarter inch crosswise slices. These were cooked in boiling, distilled water for three minutes.

Kale. Kale was purchased in bushel lots weekly. Stems and dead leaves were discarded and the remaining edible portions washed in tap water, rinsed in distilled water, and cooked by steaming for 10 minutes.

Milk. Spray-dried whole milk solids were used without further preparation.

Hydrogenated fat.[6] This was purchased as needed and used without further preparation.

After cooking, the foods were spread thinly on trays and dried in a dehydrator with forced draught at 70°C. When

[4] Packaged under nitrogen and conforming with the specifications of the U. S. Quartermaster Corps.

[5] Identified by the Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture.

[6] Crisco.

drying was complete, the products were "conditioned" by exposure to the air in thin layers for two hours, with frequent stirring; they were then finely ground, placed in airtight containers and stored at 4°C. until needed. The storage time was never longer than two weeks. The ground foods were combined by thorough mechanical mixing. Sodium chloride and viosterol were added at levels of 1 gm and 0.03 ml per 100 gm of completed diet, respectively.

The form of the food upon which to base the calculation of the amount of each dried product to be combined in the dietary mixture was selected according to the reproducibility of its weight, and was different for each food; e.g., the freshly-baked, cooled loaf for bread; dried beans, dried egg, and dried milk as they came from the container in which they were purchased; potatoes, after cooking and peeling; raw, edible portions of carrots and kale after washing and removal of excess moisture; beef and pork, prepared for roasting. Each lot of food was weighed first at the stage indicated above and again just before grinding. Allowance was made for bone and excess fat removed from the meat after cooking. The factor $\frac{\text{final weight}}{\text{original weight}}$ was used to calculate the amount of dried product which represented the required amount of undried food. The factors obtained in this way were remarkably constant for a given food in spite of the many variable conditions encountered in purchase and preparation.

Analysis of diets

Representative samples of the diets were analyzed for total nitrogen by the Kjeldahl procedure; for fat by extraction with diethyl ether in the Bailey-Walker apparatus; for thiamine by the thiochrome method of Connor and Straub ('41) with minor modifications; for riboflavin and niacin by the microbiological methods of Snell and Wright ('41) and Snell and Strong ('39), respectively; for vitamin A by bioassay. Calcium was determined by the permanganate titration method (Association of Official Agricultural Chemists,

'40) and phosphorus by the molybdate volumetric procedure (Association of Official Agricultural Chemists, '40).

Management of animals

Both male and female rats from the laboratory breeding colony were placed on the experimental diets at weaning, when they were 21 to 28 days of age and weighed 39 to 49 gm. The group that received each experimental diet consisted of 10 males and 20 females; 10 females were mated at maturity, and their reproduction and lactation performance studied; growth and food consumption, uncomplicated by reproduction and lactation, were studied in the remaining 10 females. Litter mates were assigned to diets 1 and 2 and the laboratory stock diet,[7] which was included in this series as a standard of reference; litter mates were likewise assigned to diets 3, 4, and 5. The present report is concerned with the male and unmated female animals only; the reproduction and lactation studies will be reported at another time.

All animals were maintained in individual metal cages on large-mesh raised screens in an air-conditioned laboratory at 25.5° to 27.8°C. and a relative humidity which, although not rigorously controlled, averaged 40%, with only minor variations. Food and distilled water were given ad libitum; food spillage was discouraged by the use of metal inserts in the jars when necessary; food intake was measured three times weekly, and care was taken to collect and weigh the scattered food.

All animals were weighed at least twice weekly for the first month and then once a week until the end of the experiments. Vaginal opening time was noted in the females

[7] Whole ground yellow corn 20%, whole pulverized oats 16.25%, whole ground wheat 20.5%, linseed oil meal 5%, soybean meal high temperature process, containing not less than 44% protein 5%, dehydrated beef meal 5%, beef liver meal 2%, powdered American cheese 4%, skim milk powder 7%, dried cheese whey 3%, alfalfa leaf meal 10%, irradiated yeast 0.25%, iodized NaCl 0.5%, $CaCO_3$ 1%, bone meal 0.5%, manganous sulphate 36.8 gm per ton, wheat germ oil 8 oz. per ton.

and observations made on the appearance of abnormalities such as colds, body sores, alopecia, and tumors.

At approximately 500 days of age the animals were opened under sodium amytal anesthesia, the liver was removed for analysis, the amount of body fat estimated, and the organs examined for gross signs of pathological conditions. Results of the chemical examination of the livers will be published separately.

RESULTS AND DISCUSSION

The results of the analyses of the diets are summarized in table 2, and the physiological responses of the animals in tables 3 and 4.

TABLE 2

Nutrient content of 100 gm of experimental diets as fed

NUTRIENT	DIET					
	1	2	3	4	5	Stock
Protein, gm N × 6.25	11.4	10.9	20.9	18.1	17.5	24.0
Fat, gm	6.5	4.7	9.7	12.4	11.2	4.5
Carbohydrate,[1] gm	62.0	68.0	51.0	54.0	55.0	48.0
Calcium, gm	0.138	0.166	0.272	0.268	0.236	1.5
Phosphorus, gm	0.358	0.222	0.375	0.305	0.293	0.9
Vitamin A, I.U.	350	350	540	635	635	880
Thiamine, mg	0.285	0.224	0.289	0.292	0.297	0.50
Riboflavin, mg	0.507	0.382	0.883	0.636	0.610	0.99
Niacin, mg	3.34	1.30	4.24	4.52	4.60	3.3
Fuel value, cal.	350	360	370	400	390	330

[1] Average value from food composition tables (U. S. Bureau of Human Nutrition and Home Economics and National Research Council, '45).

Growth

The average weight gains made during the 10 weeks following weaning indicate that all of the rats on the experimental diets, with the exception of those on diet 1, grew better than the controls given the stock diet. Differences among the average rates of gain on the several diets were evaluated statistically by Snedecor's "t" test ('48) for small samples, paired or unpaired, as the data permitted (table 3).

TABLE 3

Physiological responses of rats receiving experimental and stock diets

OBSERVATIONS	DIET					
	Stock	1	2	3	4	5
Ave. wt. gain 25–95 days[1] gm $\pm S_{\bar{x}}$						
Males	221 ± 10.1	209 ± 13.3	228 ± 7.6	240 ± 7.5	238 ± 7.1	224 ± 6.7
Females	135 ± 7.3	139 ± 5.3	147 ± 5.6	164 ± 7.4	150 ± 5.8	151 ± 5.4
Ave. food intake 25–95 days cal./100 gm body wt./day, $\pm S_{\bar{x}}$						
Males	37.4 ± 0.57	33.7 ± 0.88	33.6 ± 0.99	29.3 ± 0.59	33.6 ± 0.63	33.5 ± 0.64
Females	39.8 ± 0.81	33.1 ± 1.30	34.7 ± 1.09	27.6 ± 0.82	32.9 ± 1.13	33.2 ± 1.04
Maximum wt. gained gm $\pm S_{\bar{x}}$						
Males	359 ± 16.9	397 ± 15.4	426 ± 18.9	415 ± 17.3	419 ± 15.2	375 ± 17.1
Females	214 ± 13.5	241 ± 9.6	234 ± 13.5	249 ± 8.7	230 ± 10.4	240 ± 12.4
Age at which max. wt. was reached, days $\pm S_{\bar{x}}$						
Males	427 ± 23.8	466 ± 22.8	456 ± 29.2	498 ± 11.8	470 ± 16.7	457 ± 21.1
Females	439 ± 10.0	455 ± 25.7	417 ± 31.1	461 ± 15.9	475 ± 24.3	483 ± 13.8
Age of vaginal patency in ♀ days $\pm S_{\bar{x}}$	46.9 ± 1.43	42.2 ± 2.64	41.4 ± 1.46	44.8 ± 1.99	45.3 ± 3.14	52.1 ± 3.20
No. of deaths before 500 days						
Males	3	0	2	0	0	1
Females	5	2	2	0	1	0

[1] The "t" values (one asterisk indicates significance at 5% and two asterisks at 1% level): 1 vs. 3 = 2.16* for ♂, 4.30** for ♀; 3 vs. 5 = 2.55* for ♂, 1.91 for ♀; 1 and 2 vs. 3 and 4 = 1.98 for ♂, 2.29* for ♀; stock vs. 3 and 4 = 1.86 for ♂, 2.57* for ♀.

Weight gains of both male and female experimental animals were greatest on diet 3, which contained the smallest proportion of cereal and the largest amount of egg, and least on diet 1, in which 65% of the total calories were derived from corn meal. Females on diets 1 and 2 combined in one

TABLE 4

Incidence of pathological lesions in rats on experimental and stock diets

DIET AND TOTAL NUMBER OF ANIMALS	NO. ANIMALS NOT AUTOPSIED [1]	NUMBER ANIMALS SHOWING:							
		Body fat		Body sores	Malocclusion	Nephrosis	Bronchiectasis [2]		
		+ to ++	+++ to ++++				0	+	+++
Stock									
10 ♂	2	7	1	4	0	1	8	0	0
10 ♀	3	7	0	2	1	0	5	2	0
1									
10 ♂	0	2	8	9	0	2	9	1	0
10 ♀	2	3	5	7	0	0	3	5	0
2									
10 ♂	2	2	6	8	0	0	5	1	2
10 ♀	2	2	6	6	0	1	5	2	1
3									
10 ♂	0	2	8	7	6	2	1	4	5
10 ♀	0	7	3	8	0	1	2	3	5
4									
10 ♂	0	5	5	8	7	1	2	3	5
10 ♀	0	5	5	6	1	0	1	5	4
5									
10 ♂	1	2	7	8	2	1	1	6	2
10 ♀	0	5	5	9	0	0	4	3	3

[1] Animals found dead, too decomposed for autopsy.

[2] Zero = no lesions; + = small, scattered lesions; +++ = large consolidated areas.

group gained weight significantly more slowly than those on diets 3 and 4 combined, and a similar difference between the corresponding sets of males fell just short of significance. Diet 5 was omitted from this comparison because it showed a significant difference from diet 3. These differences in weight gains could not be attributed to differences in caloric intake,

since the rats making the largest gains actually consumed the fewest calories per unit of body weight (table 3).

The experiences of a number of investigators who have attempted to appraise human-type diets (including those of U. S. wage earners in cities, army rations, and war-time European and Chinese diets) by animal experiments have recently been summarized (Williams, '47), and afford a considerable body of data and discussion with which to compare our results. Winters and her co-workers (Hemphill, Koenig, and Winters, '43; Williams, '47) reported satisfactory growth in rats fed a natural cooked food diet, and the same diet diluted with one-sixth and one-third cereal, respectively. The ability of the diet to promote growth was affected neither by these changes in composition nor by addition of a vitamin and mineral enrichment mixture. Additional cereal, amounting to 57% of the dietary calories, led to diminished growth, which was not improved by enriching the cereal. Vitamin-free casein at a 10% level, however, improved growth considerably. In the present study, 65% of the caloric value of diets 1 and 2 was supplied by cereal, apparently without marked deleterious effect on growth.

Taylor (Williams, '47), in comparing the early growth of rats on diets patterned after a "low-cost, well-selected diet," and after a number of typical restricted diets, found that even the best of these was inferior to Sherman's well-known diet 13.[8] The growth of the rats on the diets used in the present study, however, surpassed that reported by Taylor for animals on diet 13.

Sperling, Loosli, Barnes and McCay ('46) fed rats a basal diet (A), made up of 14 natural foods. Growth was definitely retarded, and was improved by fortification of diet A either by (1) increasing the amounts of milk and carrots and adding liver and alfalfa leaf meal, with a corresponding reduction in the potatoes and sugar, or (2) adding a concentrated vitamin

[8] Diet composed of two-thirds whole wheat, one-third dried whole milk, and NaCl equal to 2% of weight of wheat.

mixture. Weight gains were still lower than those achieved by the animals in our study.

Maynard (Williams, '47) attributed the inferior weight gains of the animals studied by Sperling et al. ('46) partly to the large bulk and hence low caloric concentration of their diets, which suggests that moisture was not removed from the cooked foods before feeding. This would not appear to be the chief limiting factor, however. Winters demonstrated that although there was no difference in caloric intake between two groups of rats, one of which received a dry stock diet and the other a moist mixture of cooked natural foods, nevertheless the animals on the latter diet did not grow as well as those on the stock diet.

Pertinent observations on the ability of the adult rat to adapt its caloric intake to dilution of dietary nutrients by greater food consumption have been made by Adolph ('47) and by Janowitz and Grossman ('49).

Adolph studied the ability of the rat to adapt its food intake to caloric needs when a concentrated diet was diluted with kaolin, cellulose, or water, and found that its alimentary capacity allowed this animal to handle approximately one-fourth of its body weight as bulk (indigestible solids and water) and one-fourteenth of its body weight as roughage, daily; 5 times as much total ingesta could be taken when the bulk was mainly water. Janowitz and Grossman reported similar results, but did not believe that the degree of dilution at which failure of caloric adjustment occurred was determined by the rat's alimentary incapacity.

The relatively poor animal growth on human-type diets reported in the Williams symposium ('47) was generally attributed by a majority of the investigators to a content of dietary protein insufficient for the requirements of rapidly-growing young rats. The composition of our experimental diets (table 2) indicates that low protein can be only partially responsible for the growth differences observed; the natural-food diets producting the best growth (3 and 4) contained 18 to 21% protein by weight, and those producing poorer

growth (1 and 2) had only 11% protein. The stock diet, however, with 24% total protein, induced smaller gains than diets 3 and 4 and no greater gains than diets 1 and 2. The sources of the dietary protein may provide an explanation for the apparent inconsistency.

Goettsch ('48) has recently shown that only 14.3% protein (by weight), derived from a rice-beans-casein mixture in which more than 50% of the protein was supplied by casein, sufficed to promote optimum weight gains in young rats.

In our experiments diets 1 and 2 and the stock diet derived 35, 37, and 33% of their protein, respectively, from animal sources; in contrast, 63 and 51% of the proteins of diets 3 and 4, respectively, were of animal origin.

The maximum body weights attained by our experimental animals did not vary greatly with the diet fed, although the stock ration promoted smallest ultimate, as well as smallest early, weight gains. Furthermore, the animals which ate the stock diet ceased to gain weight at an earlier age than did the groups which received the experimental diets. The contrast between diet 3 and the stock diet was especially marked; many of the males on diet 3 were still gaining at 500 days when they were killed, and how much heavier they would have grown can only be conjectured.

The experiments of Sperling et al. ('46) are the only ones that have come to our attention that were continued for long enough to produce comparable data. The maximum weights reported by these investigators were considerably less than the ones given here, in spite of the similarity between the food plans of the two studies.

Sexual maturity

Sexual maturity in all groups of females, as evidenced by vaginal patency, occurred within the normal age range characteristic of the breeding colony of this laboratory. The vaginal orifice opened from 6 to 8 days later in the animals which received the stock diet and diet 5 than in their respective litter mates. These two diets differed from the others in

that neither contained egg. It is of interest that egg yolk (Marlow and Rickert, '40; Ogihara, '37) has been reported to possess estrogenic hormone sufficient to influence sexual physiology in the rat when egg is included in the diet, even in relatively small amounts.

Mortality

Fourteen deaths occurred among the 120 animals before the end of the 500-day experimental period. Acute respiratory infection, as evidenced by loss in weight, bloody nose, and labored breathing, was the cause of death in three of the animals on the high cereal diets, three on the stock diet, and two on the diets which were lower in cereal content. In addition, three animals in the high cereal group died following glucose tolerance tests; one animal on the stock diet died, having labyrinthitis, one starved to death following malocclusion of the incisors, and one died from no ascertainable cause.

Body fat

The amounts of visceral fat, estimated by visual inspection at autopsy, varied markedly among the several dietary groups. In the stock group, only one male had excessive fat and the rest had accumulated small to moderate stores. The human-food diets, on the other hand, promoted large to excessive amounts of visceral fat in from one-half to two-thirds of the animals.

Teeth

Greatly elongated upper incisors, resulting from malocclusion, appeared in about two-thirds of the males fed diets 3 and 4. The incidence of this defect was negligible in the males of the other dietary groups and in the females on all diets. There was no depigmentation of the teeth.

Malocclusion of the incisors, either with or without depigmentation, has been produced in rats by a variety of dietary means, including: addition of 226 parts per million of fluorine to an otherwise adequate diet (McCollum, Simmonds, Becker

and Bunting, '25); border-line intakes of vitamin A (Wolbach and Howe, '33; Callison and Knowles, '45); deficiency of Ca and P (Gaunt, Irving and Thomson, '39); and army ration C, both with and without cod liver oil supplements (Williams, '47).

An explanation of the tooth abnormalities observed in the studies here reported by obvious mineral or vitamin imbalances would be gratifying but unjustified on the basis of the dietary analyses. Malocclusion occurred almost exclusively in the male animals on the diets relatively highest in Ca and P and with the Ca : P ratios most nearly adequate for normal calcification; the vitamin A content of these diets was intermediate in the series (table 2). No fluorine analyses were made.

Skin

Poor skin condition was evident in many of the animals fed the human-food diets. The hair was thin or entirely lacking in limited areas and, in addition, large body sores appeared in various locations: shoulders, back, neck, tail. Alopecia and body sores are often seen in old rats and are usually considered a sign of senility rather than of any specific disease or dietary deficiency. However, in these experiments the lesions manifested themselves at an unusually early age, especially in the animals which received the diets of higher caloric content; e.g., at an average age of 122 days for diets 3, 4, and 5, and 322 days for diets 1 and 2. Skin lesions in the stock diet controls were much fewer and less severe and they appeared much later, the earliest at 464 days of age.

Thin hair and alopecia in rats have been reported by Taylor (Williams, '47) using several army rations; by Higgins, Williams, and Mason ('43) with human-type diets low in thiamine and containing white bread but not whole wheat bread; and by Goettsch ('48) with diets composed in part of cooked human foods and varying in protein content. The latter observed that alopecia increased with decreasing protein, but that protein appeared not to be the sole factor.

Skin sores about the head have been reported by Horwitt, Hills, Harvey, Liebert, and Steinberg ('49) in rats given a diet riboflavin-deficient for human adults. Higgins mentions specifically that cutaneous lesions did not occur in his rats, although they were apparently not receiving optimum amounts of riboflavin. Lack of this vitamin cannot be the sole cause of skin sores in our animals, since the incidence was highest on the diets richest in riboflavin; in no case was the riboflavin intake less than adequate for growth and health in the non-pregnant, non-lactating rat (Griffith and Farris, '42).

Neoplasms

Only two neoplasms were observed, both in male animals fed diet 3, on the lip and in the neck region, respectively. They were not studied microscopically. Although mammary tumors of a benign character occur with moderate frequency in the females of this strain of rats, none were observed in the animals fed the experimental diets.

Kidneys

Few of the kidney abnormalities which have been reported to occur with great frequency in older rats (Saxton and Kimball, '41) were observed in these animals, and when observed they appeared in random fashion throughout the experimental groups. The gross signs of yellow-brown color and fine surface irregularity, sometimes accompanied by renal enlargement, which were observed fitted the description of the condition called nephrosis by these investigators.

Bronchiectasis or chronic pneumonia

This disease, studied extensively (Moise and Smith, '30; Passey, Leese and Knox, '36; Saxton, Barnes and Sperling, '46; Smith, Bethune and Wilson, '30), is probably the most prevalent chronic illness found in old laboratory rats. Typical lesions were observed in the lungs of many of our experi-

mental animals. They varied in severity from small, scattered, grayish areas characteristic of an early stage of the disease, to large accumulations of caseous material which frequently involved as much as three-fourths of the lung tissue. Diet appeared to influence the development of this condition. It was practically absent in the whole group of stock controls which lived to 500 days; only two females showed signs of mild bronchiectasis. The incidence of mild and moderate bronchiectasis was higher on diets 1 and 2; only one male, on diet 2, had massive lesions. In marked contrast, very few animals on diets 3, 4, and 5 were free from the disease, and many had advanced lesions.

Saxton and associates (Saxton, '45; Saxton, Barnes and Sperling, '46) have likened bronchiectasis in the rat to arteriosclerosis in man, in that they are both chronic, progressive diseases which limit the life span in their respective species. Tissue elasticity is impaired in both diseases. After a study of many food combinations, these workers concluded that there was no difference between high and low protein intakes, between casein and liver as protein sources, or between exercise and no exercise in their tendency to modify the development of bronchiectasis. Restriction of caloric intake was the only measure which delayed its appearance, presumably by maintaining the body tissues in a youthful condition.

Composition of diet related to physiological observations

Study of the nutritive quality of human diets by means of the laboratory rat is not a novel procedure. More than 20 years ago McCarrison ('27) and, a few years later, Guha ('34) produced in rats symptoms of the dietary deficiencies which they regarded as characteristic of human population groups in several parts of the world by feeding the diets commonly eaten in the respective regions.

Orr, Thomson and Garry ('35) reported poor growth, nonthrifty appearance, and poor reproduction, rather than spe-

cific deficiencies, occurring in the same species on a diet approximating that of an average working class community in Scotland. The efficacy of whole milk and greens fed ad libitum in correcting the condition was shown by Gaunt, Irving and Thomson ('39) to be attributable to their calcium and phosphorus content. Higgins and associates ('43) produced impaired growth rates by feeding rats a human-type diet deficient in thiamine, but reported no specific thiamine deficiency symptoms; riboflavin, in addition to thiamine, was necessary to restore normal growth.

The inherent complexity of natural food, human-type diets gives rise to difficulty not only in producing specific pathological manifestations characteristic of well-defined deficiency, such as occur with semi-purified diets, but also in relating the observed symptoms to their probable dietary cause with any degree of confidence.

In the present study, although the same difficulties were encountered, there was evident a general pattern of physiological response to the diets used. It is significant that the stock diet, which promoted the smallest early weight gains, also gave adult animals of lowest maximum weights and in the best physiological condition, in contrast with the fast-growing young animals on human-food diets 3 and 4, which attained the highest maximum weights and were inferior in physiological condition, as judged by the high incidence of severe bronchiectasis and poor skin condition. These observations support Mayer's ('49) statement that "a diet promoting good economical growth in youth tends to promote fat deposition in adulthood, and possibly the development of degenerative diseases," and that it is dangerous to assume that diets judged adequate in growth studies are "good" maintenance diets. The weight gains of adult rats have been shown to consist predominantly of fat (Pickens, Anderson and Smith, '40). Indeed, Mayer ('48) has characterized the physiological transformation which occurs with aging of the animal body as essentially a shift from protein to fat syn-

thesis. Saxton ('45) has listed a thin body as one of the factors contributing to longevity.

Differences in fat content among the diets, although not large, appeared to be most closely related to the physiological differences observed (table 2). Higher fat intakes seemed consistently to be correlated with excess body fat, bronchiectasis, and skin lesions (table 4), although fat is frequently included in experimental rat diets at a higher level than any encountered here. It is suggestive that the blame for degenerative diseases in human beings has also been laid at the door of dietary fat (Rosenthal, '34).

It is evident that the application of the results of animal studies to recommendations for human dietary practices should be undertaken with caution, and the task of interpreting experimental data in the light of human needs should be kept in mind in planning such studies (Keys, '48).

Due allowance should be made for differences in the physiology of the digestive and metabolic processes, as well as the relative physiological ages of the two species. Although three years in the life of a rat corresponds chronologically to 90 years in the life of a man, the intermediate time intervals are not physiologically equivalent (Brody, '45). Puberty occurs in man when he has already grown to about two-thirds of his adult size; in the rat this period is reached when the animal has attained about one-third of its mature weight.

Asdell ('46) has stated that it is unsafe to attempt to equate life span data over the whole life period except when the species being compared are similar in zoologic position and habits, and that man is a particularly difficult species to fit into any system of estimating physiological age. Allowance must also be made for both the qualitative and quantitative differences in dietary needs between man and rat. For example, Scott and Norris ('49) attempted to use the rat to test foods for their value in infant feeding. Neither natural human milk nor an artificial human milk on which infants grew well proved to be nutritionally adequate for the young rat. Two factors contributed to the failure of this method of

assay: human milk contains more lactose than can be tolerated by the rat, and it is too low in protein and an unidentified "factor S" to support rat growth. In addition, the physiological age of the 4-week-old-rats was more nearly equivalent to that of a three-year-old child than that of an infant.

Finally, there may be chronic and degenerative diseases which do not have the same symptomatology and yet may be analogous in their respective species. Saxton has likened chronic bronchiectasis in old rats to arteriosclerosis in human beings, as previously discussed. Such diseases in the two species, if shown to be influenced by the same underlying fundamental dietary pattern, could be useful tools in the study of aging.

SUMMARY

In comparing the nutritive quality of certain diets, laboratory rats of both sexes were maintained on experimental rations composed of foods cooked as for human consumption. Five diets were studied in this manner, and a commercial ration used in the rat breeding colony was fed as a standard of reference.

The diets were analyzed for protein, fat, thiamine, riboflavin, niacin, vitamin A, calcium and phosphorus. Data on growth, sexual maturity, and the physical condition of the animals are presented, discussed, and compared with those of other investigators who have studied food combinations of the same nature.

It was possible to furnish 65% of the total calories as either corn meal or rice without seriously impairing the ability of the diet to support good early growth in young rats. In general, the diets that promoted the largest early weight gains and the heaviest adult animals also tended to promote deposition of body fat, a higher incidence of body sores appearing at an earlier age, and a higher incidence of bronchiectasis.

The experimental data indicate that the over-all nutritive value of human-type diets for human beings cannot be assessed by their effect on rats without careful evaluation of

the data. It is suggested that this evaluation should take into account (1) the differences in anatomy and physiology between the two species; (2) the equivalent physiological ages of the experimental rats and the human beings to which the observations are applied; (3) the comparative dietary requirements of each species; and (4) the degenerative diseases which are analogous in the two species and, though not identical, may be produced by the same fundamental dietary pattern.

ACKNOWLEDGMENTS

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EFFECT OF VARIOUS LEVELS OF CALCIUM, PHOSPHORUS AND VITAMIN D INTAKE ON BONE GROWTH

I. FOXES

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SIX FIGURES

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The minimum calcium requirement of growing fox pups, when the phosphorus (0.52%) and vitamin D (1.29% cod liver oil containing 400 I.U. vitamin D per gram) levels are held constant, has been determined to be not less than 0.51% in the dry diet (Harris et al., '45). No information on the phosphorus requirement of these animals is available. Similarly, no one has determined the vitamin D requirement of growing foxes, although Smith and Barnes ('41) found that 95 I.U. of vitamin D per fox per day resulted in slight bone healing in one rachitic fox pup, whereas 100 and 200 I.U. of vitamin D per fox per day gave good healing in two pups when the diet contained 0.10% of calcium and 0.52% of phosphorus.

Furthermore, the correct ratio of calcium to phosphorus in a diet, necessary for the normal growth and well-being of

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[2] At the time this work was conducted the research concerned with foxes was under the Fish and Wildlife Service, United States Department of the Interior. Effective June 30, 1946, all activities pertaining to fur farming were transferred to the United States Department of Agriculture.

young foxes, is unknown. Ott and Coombes ('41) produced rickets in fox pups fed a vitamin D-low basal diet when the calcium to phosphorus ratio was 7:1, although other foxes fed a ration containing 7 times the usual amounts of these two elements in the ratio of 1.32:1 were normal.

Smith and Barnes ('41) produced rickets in fox pups fed a diet low in vitamin D and containing either 0.10% of calcium and 0.52% of phosphorus or 1.64% of calcium and 0.54% of phosphorus. Their data also support the hypothesis that if fox pups are fed a diet adequate in the quantity of calcium and phosphorus and containing these elements in the ratio of approximately 1:1, rickets will not be produced, but to produce optimum bone growth some source of vitamin D is required.

The requirements for and the utilization of calcium, phosphorus and vitamin D depend upon the concentration of each in the diet, the ratio of calcium to phosphorus, the presence or absence of vitamin D or its equivalent in ultraviolet irradiation, and the rate of food consumption, and hence the rate of growth. Other factors, such as the intestinal reaction, the acid base balance of the diet, and various concentrations and ratios of other nutrients, such as magnesium and vitamin A, are also important.

It is thus apparent that one should know the effects of feeding simultaneously different levels and ratios of calcium, phosphorus and vitamin D. It would seem that the amounts fed experimentally should be at levels marking the best physiological efficiency, at levels below this range, and at levels above this range. The data of the experiment herein reported cover part of this range.

EXPERIMENTAL

One hundred and twenty-eight fox pups, equally divided as to sex, were allotted by chance to the 64 treatments outlined in table 1. To prevent age from influencing the results, all animals were started on experiment at 50 days of age, which is the usual time of weaning, and killed by electrocution at 254

TABLE 1

Experimental design[1] *and deficiency symptoms*[2]

I.U. VIT. D PER KG BODY WT.	SEX	% P / % Ca	0.3				0.4				0.6				0.8			
		Ca : P	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0
			1.0	1.7	2.0	3.3	0.75	1.2	1.5	2.5	0.5	0.83	1.0	1.7	0.38	0.62	0.75	1.2
Basal	♀		G	F	G	F	B	D	A	E	G	O	O	O	G	G	O	O
diet	♂		D	G	G	C	G	O	A	E	G	O	O	O	I	O	O	O
15	♀		G	D	F	C	G	D	D	I	G	A	O	O	E	J	A	O
	♂		H	G	E	E	G	O	O	G	D	A	O	O	I	E	D	O
50	♀		H	G	G	G	H	O	G	I	G	A	O	O	E	B	O	O
	♂		F	G	J	I	G	F	F	D	G	O	O	O	G	O	O	O
200	♀		G	G	I	K	H	O	O	F	E	O	O	O	I	O	O	O
	♂		G	G	I	K	H	O	O	F	E	O	O	O	I	O	O	O

[1] A male and a female were allotted at random to each of the 64 treatments.

[2] Letters indicate deficiency symptoms as follows:

O, Normal
A, Lameness
B, Crooked legs
C, Death, lameness, crooked legs, x-ray
D, X-ray
E, Lameness and crooked legs
F, Crooked legs and x-ray
G, Lameness, crooked legs, and x-ray
H, Lameness, crooked legs, x-ray, enlarged joints
I, Lameness, crooked legs, recurrent spasms, x-ray
J, Crooked legs, x-ray, enlarged joints
K, Lameness, crooked legs, x-ray, cranial enlargement.

days of age, at which time all pelts were prime and ready for removal.

All animals received a basal diet of the following percentage composition: dried blood fibrin 8.0, fresh frozen horse muscle meat 10.0, fresh blood 5.0, fresh horse or telang livers 4.0, dried brewers' yeast 1.0, cottonseed oil 0.3, wheat germ oil 0.2 (containing 0.03% of 90% beta- and 10% alpha-carotene), dry tomato pomace 0.5, bread meal 20.5, sodium chloride 0.5 and water 50.0. This basal diet contained 34.0% of dry matter. The dry matter on analysis showed the following percentage composition: protein 40.56, ether extract 5.27, ash 5.42, crude fiber 4.06, nitrogen-free extract 44.69, calcium 0.126, phosphorus 0.326, and vitamin D 0.82 I.U. per gram. The vitamin D content was biologically assayed in a separate experiment with rats.

At the time of mixing, the basal diet was divided into 16 portions and sufficient amounts of calcium carbonate and anhydrous secondary sodium phosphate were added to furnish the amounts of calcium and phosphorus outlined in table 1. Vitamin D oil [3] was administered orally by syringe immediately after each weekly weighing. Amounts allowed were based on the current week's weight, as outlined in table 1.

The foxes were housed in raised, wire-floored pens suitably darkened to keep out direct or reflected sunlight. No bedding was used. The animals were fed once daily as much of their respective diets as they would consume and records were kept of the food consumption. Fresh water was supplied daily. All animals were treated for ear mites and for ascarids at weaning and twice during the experiment. Weights were also taken at weekly intervals.

Since the foxes were not killed until they reached an age of 254 days, an x-ray photograph of the tibia-fibula of each fox was taken at approximately 150 days of age to note the bone development. These data were used, along with the deficiency

[3] The oil used consisted of A.R.P.I.-processed ergosterol, activated by low velocity electrons in vegetable oil containing 11,000 U.S.P. units of vitamin D per gram; control number D1053, lot number 3098.

symptoms, to determine the requirements of calcium, phosphorus and vitamin D during the early stages of growth.

Following electrocution, the right humerus of each animal was removed and the following weights and measurements were taken on each humerus: fresh weight, weight in water, weight of ether-extracted dried bone, weight of ash, length, bone thickness and degree of bending. The volume in cubic centimeters was obtained by calculating the weight of water displaced by the tissue, and assuming that 1 gm of water represented 1 cm^3. The bone thickness was measured at a point one and one-fourth inches from the distal end. The degree of bending was determined by placing the bone on a smooth straight surface and measuring the distance up to the height of greatest bending. Covariance analysis was used to adjust the ash content of each bone to an equal volume and an equal weight of ether-extracted dried bone. An x-ray photograph was also taken of each humerus. The other procedures employed have been previously described by Harris et al. ('45).

At pelting time the skins were objectively measured for size and the other parts were classified into 5 grades by an expert of one of the New York auction companies. The data were analyzed statistically by the methods of Snedecor ('46).

RESULTS AND DISCUSSION

The 4 percentage levels of calcium in composite samples of the dry diets were 0.342, 0.548, 0.622, and 1.04, and the 4 percentage levels of phosphorus in the dry diets were 0.326, 0.400, 0.600, and 0.759, respectively.

The level of vitamin D had little effect on the shape of the bones or on the density as determined by x-ray. However, the humeri from the foxes fed 0.3 and 0.5% calcium and 0.3 and 0.4% phosphorus were lacking in minerals, were more bent and were thicker than the humeri from those foxes fed 0.6 and 1.0% calcium and 0.6 and 0.8% phosphorus. Large amounts of calcium in relation to phosphorus or large amounts of phosphorus in relation to calcium caused a mineral poverty and thickened, bent bones.

The deficiency symptoms noted were lameness, crooked legs, recurrent spasms, enlarged joints, cranial enlargement, and a mineral poverty as noted by x-ray (table 1). These symptoms had been previously described by Harris et al. ('45), although they were less severe in the present experiment.

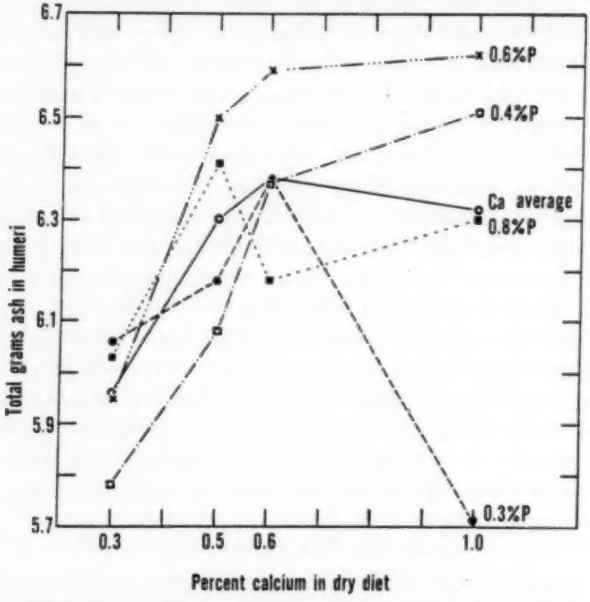

Fig. 1 The effect of calcium at various levels of phosphorus intake on the amount of ash in the humeri.

Another condition noted in some of the foxes was an undershot jaw. In some instances the mandible extended from one-eighth to one-fourth inch beyond the maxilla. Burrill ('43) has described similar protrusions of the mandible in dogs receiving diets low in phosphorus.

Space does not permit a discussion of all the measurements taken on the humeri, but the various measurements gave similar results. In general the statistical analysis of the measurements taken on the humeri showed that the difference in cal-

cium and phosphorus levels was significant but the difference in vitamin D levels was not. There was also a significant interaction between calcium and phosphorus, which supports previous views on the importance of the ratio of calcium to phosphorus.

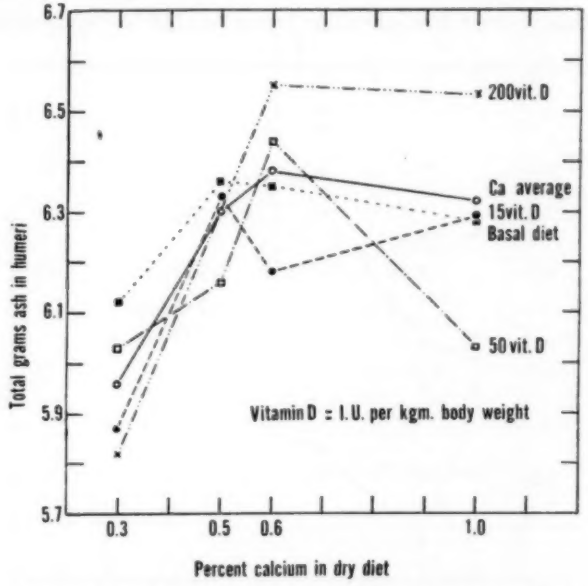

Fig. 2 The effect of calcium at various levels of vitamin D intake on the amount of ash in the humeri.

The data for the total ash in the humeri after the humeri are adjusted to a constant volume by covariance analyses are given in figures 1 through 6. This was the measure which best described the results of the humeri analyses.

After all the various measurements are taken into consideration, it is apparent that even with increasing amounts of phosphorus and vitamin D all the foxes receiving the 0.3% and some of those receiving the 0.5% calcium diet showed deficiency symptoms. Similarly, with increasing amounts of cal-

cium and vitamin D all the foxes receiving 0.3% and some of those receiving 0.4% of phosphorus in their diet showed deficiency symptoms. Those foxes, however, receiving 0.6 or 1.0% calcium along with 0.6 or 0.8% phosphorus were normal. This corresponds to a calcium-phosphorus ratio of from 1.0:1.0 to 1.7:1.0. It appears also that the optimum amount of calcium and phosphorus for growing foxes would be approximately

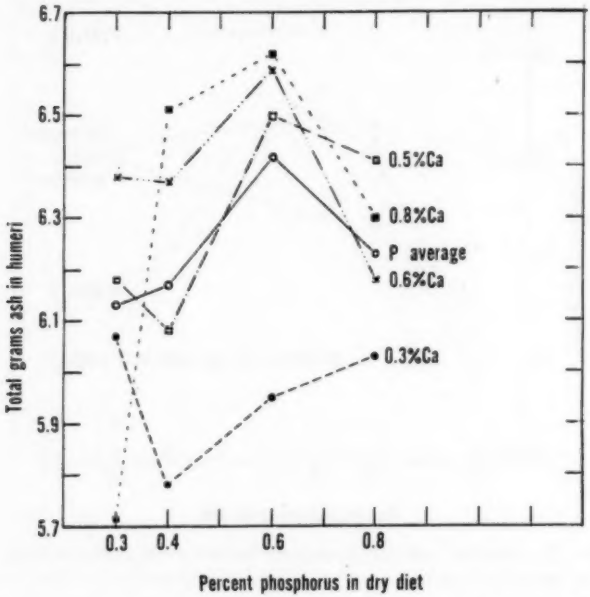

Fig. 3 The effect of phosphorus at various levels of calcium intake on the amount of ash in the humeri.

0.6% of each, which corresponds to a calcium to phosphorus ratio of 1:1. Finally, it should be noted that a phosphorus content of 0.8% seems to be approaching the upper limit for greatest physiological efficiency.

If the effect of vitamin D per se in preventing deficiency symptoms is considered, it is apparent that as the amounts of vitamin D were increased from that supplied in the basal diet

to 200 I.U. per kilogram of body weight per day there was not a corresponding decrease in the incidence of deficiency symptoms. Some foxes showed normal bones, although they received only the basal diet plus the calcium and phosphorus carriers. We are therefore inclined to conclude that the vitamin D requirement of the growing fox is less than the amount supplied by the basal diet.

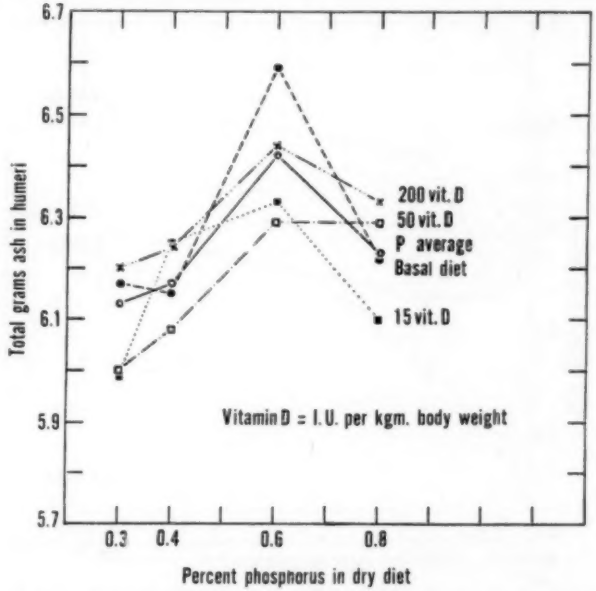

Fig. 4 The effect of phosphorus at various levels of vitamin D intake on the amount of ash in the humeri.

The humeri of male foxes had more total ash and a greater volume, and the fresh and ether-extracted dried humeri weighed more than the humeri of the female foxes. There was no measurable difference between sexes in their requirement for calcium, phosphorus and vitamin D.

In practice it would seem desirable to recommend a nutrient allowance of 0.6 to 1.0% calcium and 0.6 to 0.8% phosphorus,

with a calcium to phosphorus ratio of from 1.0:1.0 to 1.7:1.0. The amount of vitamin D in the basal diet, 0.82 I.U. per gram of dry food plus 200 I.U. per kilogram body weight, did not improve performance, but it was not toxic to the animals.

More calcium and phosphorus and less vitamin D may produce normal bones during early stages of growth, but our experiment does not go beyond the above levels. Also, since the

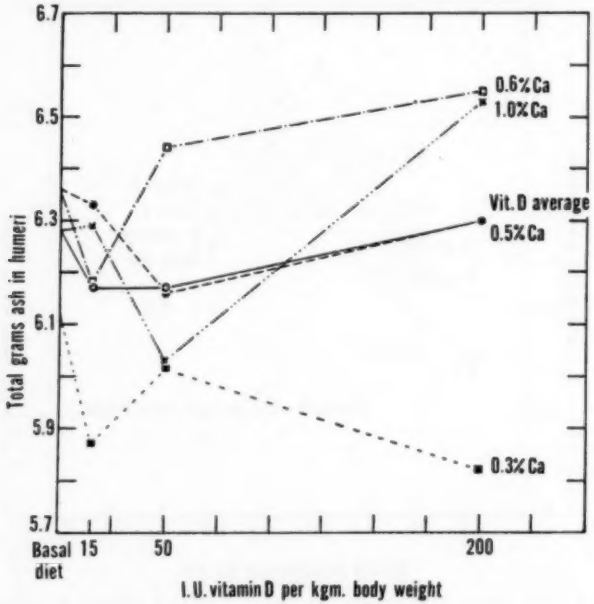

Fig. 5 The effect of vitamin D at various levels of calcium intake on the amount of ash in the humeri.

foxes were kept on the same diets until they reached an age of 254 days, it is possible that less calcium, phosphorus and vitamin D could be fed toward the end of the growth period.

Until deficiency symptoms developed, the foxes gained at a rate of 0.6 to 0.7 lb. per animal per week, which is above the normal rate of 0.5 lb. per fox per week as determined for the

foxes at this station. This was gratifying, because in many experiments of this nature the animals gain at a subnormal rate, and under these conditions the requirements would be less than they should be for a rapid rate of gain.

The various levels and ratios of calcium, phosphorus and vitamin D that produced normal bones also produced normal pelts.

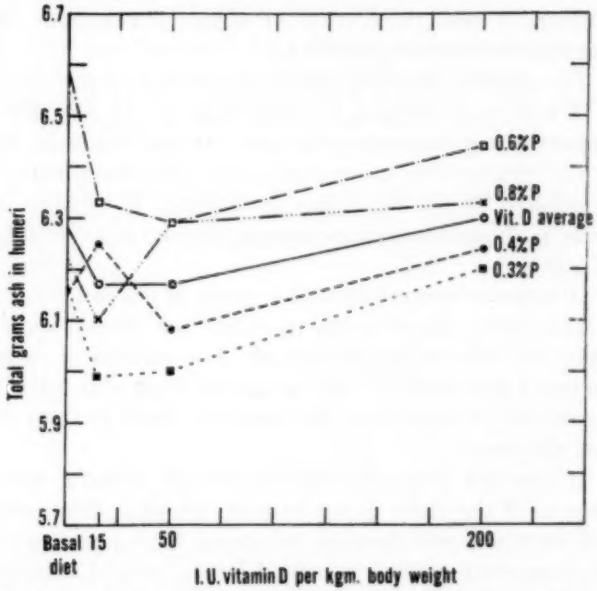

Fig. 6 The effect of vitamin D at various levels of phosphorus intake on the amount of ash in the humeri.

SUMMARY AND CONCLUSIONS

In a factorially designed experiment the effects on bone growth of 4 levels of calcium, 4 levels of phosphorus, 4 levels of vitamin D, and of sex were investigated. One hundred and twenty-eight foxes were fed the experimental diets, beginning at a weaning age of 50 days and continuing to the time of pelting at an age of 254 days. X-ray photographs of the tibia-fibula were taken at about 150 days of age.

At the close of the experiment the following measurements were taken on the isolated humeri: weight of ash, volume, weight of ether-extracted dried bone, length, bone thickness and degree of bending. Covariance analysis was used to adjust the ash content of each bone to an equal volume and an equal weight of ether-extracted dry bone. The ash per unit volume after covariance analyses best described the results on the humeri. Under the conditions of this experiment, the following conclusions are justified:

1. The calcium requirement of the growing fox at an age of 50 to 254 days is between 0.5 and 0.6% in the dry diet. The phosphorus requirement is between 0.4 and 0.6% in the dry diet. The vitamin D requirement is less than the amount which was supplied by the basal diet employed. It appears that a calcium to phosphorus ratio ranging from 1.0:1.0 to 1.7:1.0 is satisfactory.

2. In practice one could feed a range of 0.6 to 1.0% calcium and 0.6 to 0.8% phosphorus, provided the correct calcium to phosphorus ratio were maintained. The amount of vitamin D in the basal diet, 0.82 I.U. per gram dry food plus 200 I.U. per kilogram body weight, was not above the limit for best physiological efficiency.

3. If the diet does not contain enough calcium and phosphorus, or if the ratio is not correct, growing foxes about 50 to 100 days old will develop lameness, bent and crooked leg bones, recurrent spasms, enlarged joints, cranial enlargement, and malformed body bones.

4. The humeri of male foxes at the end of the experiment had more total ash and a greater volume, and the fresh and ether-extracted dried humeri weighed more than the humeri of the female foxes. There was no measurable difference between sexes in their requirement for calcium, phosphorus and vitamin D.

5. The time-saving value of the factorial design of the experiment is evident from its use here in arriving at these measurements of the calcium, phosphorus, and vitamin D requirements of the growing fox.

ACKNOWLEDGMENTS

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THE PROTEIN REQUIREMENT OF GROWING FOXES

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THREE FIGURES

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Fox ranchers usually feed growing pups a diet which contains 35 to 45% protein on a dry matter basis. Such a high percentage of protein appears to be considerably above the amount actually needed when compared to the requirements for growing dogs (McCay, '49). The principal source of protein in most fox diets is horse meat, which is both high priced and difficult to obtain.

If quality silver fox pelts are to be produced economically, it is essential that those who raise these animals know their protein requirements during different stages in the life cycle. Particularly is it desirable to have this information on the growing fox, since a large percentage of the pelts marketed are from the current year's crop.

An experiment was conducted to determine the protein requirements of growing foxes during two stages of growth: (1) from weaning, 50 days of age, to the end of the period of most rapid growth, 161 days of age; and (2) from 162 to 259

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[2] At the time this work was conducted the Station was under the Fish and Wildlife Service, Department of the Interior. It is now under the Bureau of Animal Industry, Agricultural Research Administration, Department of Agriculture.

days of age, when the pelts are prime. The period of most rapid growth was determined from the normal growth data of 376 foxes which were raised at the United States Fur Animal Experiment Station. The age of 161 days also coincides rather closely with the time when fox pups have completed the shedding of their puppy fur and the growth of the adult coat is well started.

EXPERIMENTAL

In 1944, 128 silver fox pups were segregated equally by sex and allotted by chance to the treatments outlined in table 1. Some animals were sacrificed at 50, 161 and 259 days of age to determine the amounts of protein stored. For those studies a "skin sample" and an "empty carcass" sample were prepared and preserved by freezing. After skinning, the pelt was scraped to remove surplus fat and ground twice through a food chopper to make up the "skin sample" for those foxes sacrificed at 50 and 161 days of age. The scrapings from the skin were added to the "empty carcass" sample. For those foxes sacrificed at 259 days of age, the right half of the scraped, dried skin was cut up in small sections and ground twice through a food chopper. The "empty carcass" sample of all foxes consisted of the whole animal, except the scraped skin and the contents of the intestinal tract. This sample was prepared by passing disjointed carcass sections through a power grinder and then through a food chopper. Corrections were made for all moisture losses. Adhering material was removed from the food chopper after each grinding and added to the sample.

Digestion and balance trials were conducted on two males and two females for series A during the periods of 50 to 161 days of age and 162 to 259 days of age, and for series B during the period 162 to 259 days of age. An attempt was made to feed all animals on the metabolism tests the same amount of food for a period of 12 days, the 5th to the 11th of which constituted the collection period. This ideal was not achieved in all cases. Iron oxide was used as a feces marker. Urine was preserved

TABLE 1

Experimental design and animal equalization [1]

YEAR AND SERIES	FROM 50 TO 161 DAYS OF AGE		FROM 162 TO 259 DAYS OF AGE			
	Protein level (N × 6.25)	No. of animals	Protein level (N × 6.25)	No. of animals		
				On experiment	For carcass analyses	For pelt studies
	%		%			
1944	17.4	16	17.4	10	6	10
	22.9	16	22.9	10	6	10
A [2]	28.8	16	28.8	10	6	10
	34.3	16	34.3	10	6	10
	40.7	16	40.7	10	6	10
1944	27.0	8	17.4	8	6	8
	27.0	8	22.9	8	6	8
	27.0	8	28.8	8	6	8
B [2]	27.0	8	34.3	8	6	8
	27.0	8	40.7	8	6	8
1944 C (control)	27.0	8	27.0	8	6	8
1945	16.4	8	16.4	8	..	8
	19.1	8	19.1	8	..	8
A	22.0	8	22.0	8	..	8
	24.5	8	24.5	8	..	8
	27.2	8	27.2	8	..	8
	33.2	8	33.2	8	..	8
	38.4	8	38.4	8	..	8
1945	27.7	8	14.1	8	..	8
	27.7	8	16.4	8	..	8
B	27.7	8	19.1	8	..	8
	27.7	8	22.0	8	..	8
	27.7	8	27.2	8	..	8
	27.7	8	33.2	8	..	8
	27.7	8	38.4	8	..	8
1945 C (control)	27.7	8	27.7	8	..	8

[1] One-half the animals were males and one-half females.

[2] Three males and three females, series A, 1944, were killed for chemical analyses at 50 days of age to estimate the chemical composition of the other foxes in series A. Animals in series A, 1944, were all fed their respective diets until they reached an age of 161 days. At this time three males and three females were killed from each group for chemical analyses. The balance of the animals were fed the same diets until they reached an age of 259 days. At this time three males and three females of each group were analyzed chemically and the pelts of 5 males and 5 females of each group were saved for pelt studies.

[3] Three males and three females were killed from series B, 1944, at 161 days of age to serve as normal controls for a comparison with the animals in series A at 161 days of age and to estimate the composition of the animals in series B and C at 161 days of age. Animals in series B and C, 1944, were all fed the same diet until they reached an age of 161 days. At this time animals in series B were shifted to various protein levels and fed until they reached an age of 259 days, at which time three males and three females of each group were analysed chemically and the pelts of 4 males and 4 females were saved for pelt studies.

by adding 60 ml of formaldehyde and 50 ml of toluene to the urine container. At the end of the collection period the washings and urine were adjusted to 6000 ml. The feces were preserved by covering them with a mixture of 97 parts alcohol and three parts hydrochloric acid.

In 1945 the 1944 experiment was repeated, with the exception of slaughter analyses and balance trials. As is shown in table 1, additional protein levels were fed. One hundred and twenty silver fox pups were segregated equally according to sex and allotted by chance to the various treatments.

The diets (table 2) were as complete in nutrient content, particularly vitamins and minerals as knowledge permitted, and contained the same amount of moisture, fat, calcium and phosphorus. Diet samples were taken every mixing day, and each day of the metabolism trials. These samples were preserved by freezing and later were thawed and composited to represent the feeding periods as well as the digestion and metabolism trials.

The pups were fed individually once a day about what they would consume of their respective diets, and were housed in raised, wire-floored pens with kennel attached. Water was kept before them at all times. Records were kept of food intake.

An average of the weights taken on two consecutive days at the start and end of the respective periods constituted the initial and final weights of each animal. Weights were also taken at the beginning and end of the digestion and metabolism trials, as well as at 4-week (1944) and two-week (1945) intervals during the experiments.

At pelting time the skins were objectively measured for size and grouped into one of 5 grades for each of the 20 factors considered in determining pelt quality. Each skin was again graded by an expert of one of the New York auction companies, who estimated its commercial value.

The data were analyzed statistically by the analysis of variance method (Snedecor, '46). All differences mentioned are statistically significant, representing odds of 19 to one or more.

TABLE 2

Percentage composition of diets[1]

DIET NUMBER	F-160	F-146	F-159	F-147	F-158	F-148	F-149	F-150	F-143a	F-143b
Year in which fed	1945	1944–45	1945	1944–45	1945	1944–45	1944–45	1944–45	1944 Control	1945 Control
% protein on dry basis	14.1	16.9	19.1	22.5	24.5	28.0	33.8	39.6	27.0	27.7
Ingredients	%	%	%	%	%	%	%	%	%	%
Tomato pomace	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	1.00	1.00
Yeast: regular	1.90	1.90	1.90	1.90	1.90	1.90	1.90	1.90	0.00	0.00
irradiated	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	0.00
Salt mixture MM-[2]	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.00	0.00
Liver A	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.00	0.00
Liver (fresh)	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	5.00	5.00
Carrots	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	4.00	2.00
Cod liver oil[3]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Meat (horse muscle)	0.00	7.04	7.04	7.04	7.04	7.04	7.04	7.04	25.00[4]	25.00[5]
Limestone	0.17	0.20	0.18	0.15	0.13	0.10	0.05	0.00	0.00	0.00
Dicalcium phosphate	1.32	1.25	1.09	0.94	0.78	0.63	0.31	0.00	0.00	0.00
Cottonseed oil	1.48	1.22	1.07	0.92	0.77	0.62	0.32	0.02	0.00	0.00
Soybean meal 45%:									2.30	
Beef meal 45%:	0.00	0.00	2.09	4.17	6.26	8.35	12.53	16.69	2.30	0.00
Fish meal 10%:										
Commercial fox feed	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	25.00	25.00
Rice (cooked, frozen, ground)[6]	78.85	77.21	72.21	67.22	62.21	57.20	47.17	37.18	0.00	0.00
Water	5.10	0.00	3.24	6.48	9.73	12.98	19.50	25.99	35.00	41.60
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1] All diets, with the exception of F-143a and F-143b, were compounded to contain 33.7% dry matter, which had the following percentage composition: fat 8.67, crude fiber 2.46, ash 6.94, calcium 1.26 and phosphorus 0.922. The control diets contained 39.3% of dry matter, which had the following percentage composition: fat 10.2, crude fiber 6.49, ash 9.67, calcium 2.78, phosphorus 1.10.

[2] Percentage composition as follows: NaCl 35, KCl 10, K_2CO_3 25, Na_2CO_3 16.8198, $MgCO_3$ 9.2, $FeC_6H_5O_7 \cdot 3H_2O$ 3.6, $CuSO_4 \cdot 5H_2O$ 0.04, $MnSO_4$ 0.28, $K_2Al_2(SO_4)4 \cdot 24H_2O$ 0.02, KI 0.01, $CoCl_2 \cdot 6H_2O$ 0.02, $ZnCO_3$ 0.01 and NaF 0.0002.

[3] Contained 3,000 I.U. of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

[4] Horse muscle 10%, lungs 10%, ground green bone 5%.

[5] Muscle meat 20%, ground green bone 5%.

[6] Consisted of rice one part, water two parts.

RESULTS AND DISCUSSION

To clarify the discussion of the results the protein levels which were fed are tabulated in table 3.

Deficiency symptoms

During the period of most rapid growth, 50 to 161 days of age, it was observed that animals on the 16.9, 19.1, and 22.5% protein (N × 6.25) levels were small and unthrifty for their age, but within 60 to 70 days after starting the animals on experiment they were again normal in appearance, although still somewhat below average in size. Some of the animals in the 16.9% protein group still carried patches of unshed puppy

TABLE 3

Protein percentage levels in dry diets which were fed in 1944 and 1945

YEAR	PROTEIN LEVELS IN EXPERIMENTAL DIETS								
	F-160	F-146	F-159	F-147	F-158	F-148	F-149	F-150	Control F-143
1944	...	17.4	...	22.9	...	28.8	34.3	40.7	27.0
1945	14.1	16.4	19.1	22.0	24.5	27.2	33.2	38.4	27.7
Average	14.1	16.9	19.1	22.5	24.5	28.0	33.8	39.6	27.4

fur on their flanks in late August and early September, 4 to 6 weeks after it should have disappeared. Failure to shed puppy fur in a normal manner is generally regarded as an indication of ill health or poor nutrition.

There were no deficiency symptoms observed in the foxes during the growth period extending from 162 to 259 days of age. Eight of the foxes died, but these deaths were not correlated with the diets fed.

Food consumption

The animals receiving the 14.1% (series B, 162 to 259 days of age) and the 16.9% (series A, 50 to 161 days of age) protein diets consumed less food than those on the other diets. There

were no significant differences in food consumption among the other groups.

Growth, 50 to 161 days of age

Data for the two years have been combined in figure 1. From these data it would appear that the fox needs somewhere

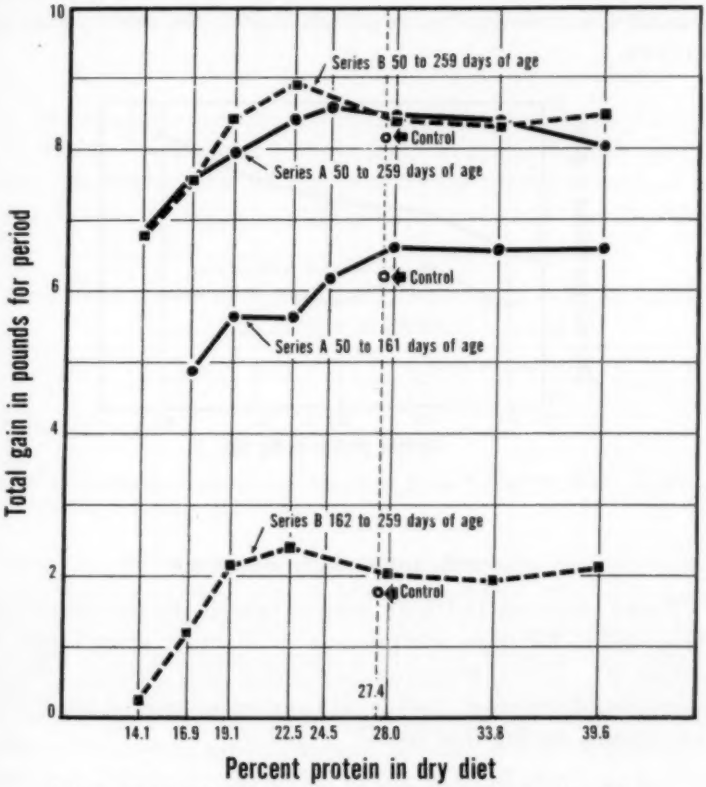

Fig. 1 Total gain of foxes when fed various amounts of protein.

between 24.5 and 28.0% protein in the diet during the period from 50 to 161 days of age.

If the gains of those foxes which were fed the same protein levels from 50 to 259 days of age are examined (fig. 1, series A),

a diet containing 24.5% protein is seen to be adequate for normal growth. There were also no differences among the foxes in body length when fed protein at this or higher levels over this period. Although underfed on protein during the period of most rapid growth, a growing fox will gain over a longer period and reach the same weight by pelting time as an animal which received more protein during the early period of growth.

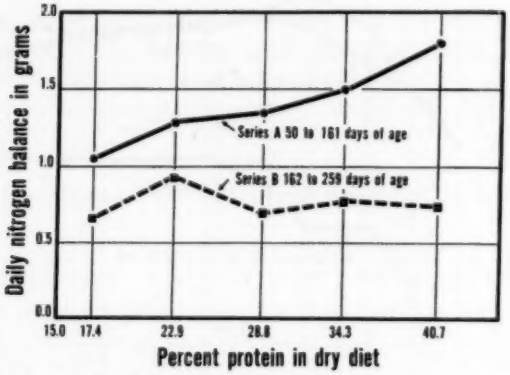

Fig. 2 Daily nitrogen balance when various amounts of protein were fed to foxes.

Growth, 162 to 259 days of age

Foxes receiving 19.1% or more of protein during the period from 162 to 259 days of age made satisfactory growth (fig. 1, series B). Those receiving less made poor growth. There were no differences in body length among animals of the different groups during this period.

Nitrogen balance

There was a highly significant linear regression for the nitrogen balance as the protein in the diet was increased from 17.4% up to 40.7% during the period from 50 to 161 days of age (fig. 2). This indicates that more than 40.7% of protein

would be required to promote maximum storage at the time the balance trials were carried out. The foxes on the lower protein diets utilized their protein to a greater extent (table 4).

During the period extending from 162 to 259 days of age it appears that 22.9% protein is near the optimum amount. This is also suggested by the growth data (fig. 1) and the amount of nitrogen stored (fig. 3, table 4).

Nitrogen storage as measured by carcass analyses

In general the nitrogen storage measurements gave the same results as the nitrogen balance test. As the protein in the diet was increased from 17.4 to 40.7%, the nitrogen storage increased for the foxes from 50 to 161 days of age (fig. 3). Thus,

TABLE 4

Per cent of absorbed nitrogen which was retained by foxes receiving various amounts of protein

AGE IN DAYS	NO. FOXES ON EACH PROTEIN LEVEL [1]	% PROTEIN IN DRY DIETS				
		17.4	22.9	28.8	34.3	40.7
50 to 161	4	45	37	32	29	27
162 to 259	4	20	22	12	13	11

[1] One-half males, one-half females.

more than 40.7% of protein would be required to promote maximum storage at the end of 161 days of age. There is some question, however, whether it would pay to feed enough protein to promote maximum storage at this age, because the foxes on less protein continued to store nitrogen over a long period of time (compare series A, 162 to 259 days of age, with series B, 162 to 259 days of age). The control foxes stored slightly more protein during the period 50 to 161 days of age than did the experimental foxes.

There were no differences in nitrogen storage during the period 162 to 259 days of age among the foxes that received diets containing 22.9, 28.8, 34.3 and 40.7% protein, but the 17.4% diet was definitely inferior. There was a tendency, how-

ever, for the foxes receiving the 22.9% protein diet to store the most nitrogen. Control foxes stored less protein during the period extending from 162 to 259 days of age than did the experimental foxes.

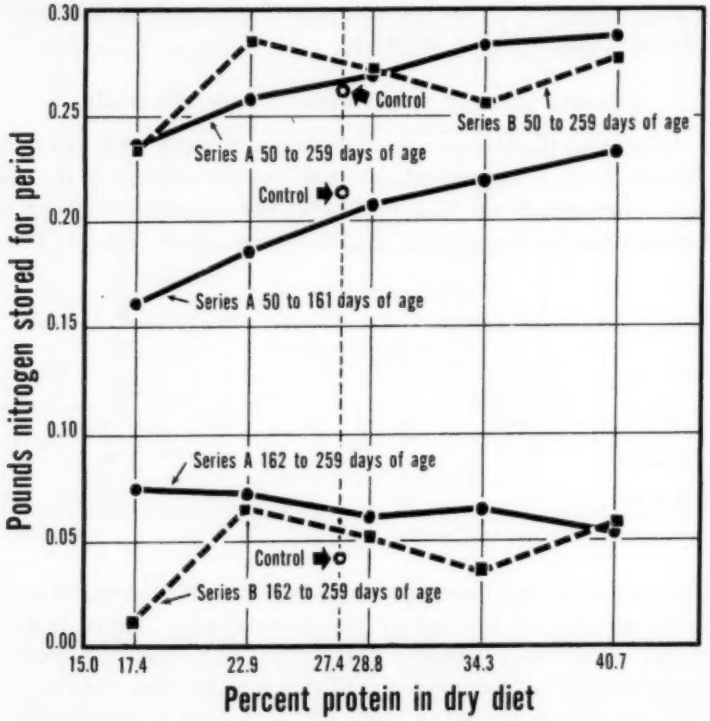

Fig. 3 Nitrogen storage as determined by carcass analysis of foxes fed various levels of protein.

Per cent of protein digested

There were no differences in the apparent digestibility of protein among the diets fed to the foxes during the period 50 to 161 days of age (table 5). There were also no differences in digestibility among the diets fed to the foxes during the period

162 to 259 days of age, although there was a tendency toward less satisfactory digestion of the 17.4% protein diet by the older foxes.

Effect of protein level on the fur

No pelt studies were made on the skins of the animals of series A, 50 to 161 days of age, either in 1944 or 1945, because growth of the adult coat of fur had just started at 161 days of age.

In 1944 the pelts of animals reaching 259 days of age (series A, B, and C) were classified into 5 different grades for each of the following factors: Primeness of leather; guard hair length, density, clarity, sheen, and texture; band-length and clarity;

TABLE 5

Apparent digestion coefficients of foxes receiving various amounts of protein

AGE IN DAYS	NO. FOXES ON EACH PROTEIN LEVEL [1]	% PROTEIN IN DRY DIETS				
		17.4	22.9	28.8	34.3	40.7
50 to 161	4	73	74	73	75	73
162 to 259	4	66	74	72	72	73

[1] One-half males and one-half females.

underfur density, length, and color; brush size, shape and color; brush tip size and color; chewing on shoulder, on hip, on brush, and on tail tip. The grading of skins for length and width was determined by measurement. Statistical analyses of these data failed to show any significant differences which could be attributed to treatment.

The 1944 and 1945 pelts of the foxes in series A, B, and C were also graded by experts of a large New York auction company. The distribution of the pelts from the different diets among the quality grades is shown in table 6. The grades of the pelts were in decreasing order from one to 8.

Good quality pelts were produced when the foxes were raised on 27.2 to 40.7% protein from 50 to 259 days of age, but diets

lower than 27.2% in protein produced poorer quality pelts during both years. It appears also that if foxes are raised on a diet containing 27.4% protein (control diet) until they reach 162 days of age, they can be shifted to diets containing 17.4 to 40.7% protein and still produce normal pelts. In other words, the level of dietary protein from 162 to 259 days of age had little effect on the fur.

The control fox pelts were definitely inferior to most of the other pelts in both years. This was gratifying because the ex-

TABLE 6

Placement or rating by fur experts on a scale of one to 8 of pelts from fox pups fed different levels of protein

YEAR	PLACEMENT OR RATING							
	1	2	3	4	5	6	7	8
	Groups identified by per cent of protein fed							
	Series A, 50 to 259 days of age							
1944	34.3[1]	40.7	28.8	22.9	17.4	...	...	...
1945	27.2	38.4	33.2	24.5	22.0	19.1	16.4	...
	Series B, 162 to 259 days of age							
1944	40.7	17.4	22.9	28.8	34.3	control	...	...
1945	19.1	27.2	24.5	33.2	14.1	control	16.4	38.4

[1] For example, the group fed a diet containing 34.3% protein in 1944 placed first, or was given a rating on 1 on the scale used.

perimental diets used were evidently of higher nutritive quality than the control diet.

Effect of sex

Male fox pups grew more rapidly and were larger than female pups at pelting time. There were, however, no significant differences in protein requirements or in the utilization of the nitrogen contained in diets of different protein content, although male foxes tended to require slightly more protein than female foxes during the early stages of growth.

SUMMARY

Two experiments were conducted with a total of 248 fox pups to determine the protein requirements of young foxes during two periods of growth, 50 to 161 and 162 to 259 days of age. Protein was fed at 17.4, 22.9, 28.8, 34.3 and 40.7% levels for all growth periods in 1944. In 1945 protein at 16.4, 19.1, 22.0, 24.5, 27.2, 33.2 and 38.4% levels was fed for the period from 50 to 161 days of age. From 162 to 259 days, a 14.1% level was added and the 24.5% level eliminated. Controls were given a standard Station diet containing 27.4% protein.

Growth, balance and carcass analyses were made for each period of growth.

The requirement of protein to promote maximum nitrogen storage in a fox pup between the ages of 50 and 161 days is above 40.7% in the dry diet, but 28% of protein produced as rapid growth as higher intakes.

It is suggested that growing foxes between 50 to 161 days of age should be given a nutrient allowance of 25 to 34% protein in their diets on a dry basis. This amount of protein is not enough to promote maximum storage of nitrogen, but foxes fed at these levels were as large at pelting time and had fully as good pelts as those fed larger amounts of protein. Since the animals are raised for their pelts only, it is possible to underfeed protein during the early stages of growth without detrimental results, if it is more economical.

Between the ages of 162 to 259 days of age fox pups should have a nutrient allowance of from 19 to 25% protein in their diets on a dry basis.

Male fox pups grew more rapidly and were larger than female fox pups at pelting time and they tended to have a slightly higher requirement for protein than females during the early stages of growth.

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THE EFFECT OF THE INGESTION OF ALCOHOL ON THE STORAGE AND EXCRETION OF THIAMINE [1]

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A few reports have been published to date which suggest that the metabolism of alcohol does not require as much thiamine as the metabolism of an isocaloric amount of carbohydrate, or that alcohol may have a "sparing" action on thiamine. Lowry et al. ('42) demonstrated that the ingestion of alcohol or whisky delayed the onset of polyneuropathy in thiamine-deficient rats. Westerfeld and Doisy ('45) found that the onset of opisthotonus and death of pigeons on a thiamine-deficient ration was delayed by the isocaloric substitution of alcohol for either fat or carbohydrate. Butler and Sarett ('48) reported that thiamine excretion increased markedly in two adult subjects after four-fifths of the carbohydrate in the diet was replaced by alcohol.

A study of the urinary thiamine and pyruvic acid excretion and the tissue storage of thiamine in paired rats with and without alcohol in the ration is reported in this paper. This work was undertaken in order to determine the effect of the ingestion of alcohol on thiamine metabolism as measured by tissue storage, thiamine excretion and the excretion of pyruvic acid.

[1] Contribution 771 of the Massachusetts Agricultural Experiment Station. Some of the data in this paper are taken from a thesis submitted by Lois E. Lloyd ('48) to the faculty of the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of Master of Science.

EXPERIMENTAL

Albino rats of the Wistar strain were used throughout the experiments. All animals were placed on experiment between the ages of 21 and 24 days, and were housed in individual wire-mesh cages on raised wire-mesh trays for the experimental period of 10 weeks. The composition of the basal ration is shown in table 1.

TABLE 1

Composition of basal ration

INGREDIENTS	PER CENT
Vitamin-free casein [1]	20
Lard	5
Corn oil	5
Salt mixture [2]	4
Cerelose (Dextrose)	66

In addition to the basal ration the following vitamin supplement was fed daily:

Vitamin	*μg*	*Vitamin*	
Thiamine	8	*p*-Aminobenzoic acid	0.5 mg
Riboflavin	10	Inositol	0.5 mg
Niacin	50	Choline	2.0 mg
Pyridoxine	15	Alpha-tocopherol	1.0 mg
Ca Pantothenate	60	Menadione	0.2 mg
Biotin	0.1	Viosterol	25 U.S.P. units
Carotene	10		

[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Phillips and Hart ('35).

This ration has been used extensively in this laboratory and has been found to promote a slow gain in weight and to cause a depletion of the thiamine stored in the body tissues (Wertz, '48). However, animals kept on this ration for as long as 10 weeks have a normal, healthy appearance, show no overt signs of thiamine deficiency and are in a subacute avitaminotic state. The assumption was made that rats in a partially depleted state would make a more sensitive test animal for this experiment than would completely deficient ones.

Series 1

Twelve pairs of weanling rats were distributed in two groups in such a way that each rat in group 1 had a litter mate of the same sex and weight in group 2. The basal ration was fed, using the "paired" feeding technique. The food consumption of each pair of rats was controlled by the intake of the pair mate that ate the least. Group 1 was fed a supplement of a 25% ethyl alcohol solution (95% alcohol and distilled water, *sp. gr.* 0.970) and group 2 a supplement of dextrose in an amount isocaloric to the alcohol consumed by group 1. As dextrose makes up 66% of the basal ration, feeding it as a supplement introduced no new food factor into the diet of group 2.

It was found in preliminary work that rats would voluntarily drink enough of the 25% alcohol solution to cause unconsciousness of several hours' duration. As this situation was not desirable, the amount of alcohol fed was limited to that amount which would cause some incoordination but only a rare case of unconsciousness. The rats in group 1 were fed 2 ml of the 25% alcohol solution daily during the first two weeks. Thereafter weekly increments of 1 ml were added, until by the 10th week the rats were drinking 10 ml daily. The daily supplement of alcohol was divided into two feedings (9 A.M. and 5 P.M.), and at first was introduced directly into the rat's mouth by pipette. As the rats rapidly developed a taste for the alcohol and it was found that they would consume it immediately from a glass supplement dish, this method of feeding was then employed.

Urine collection and analysis. In the last 10 days of the experimental period, two 24-hour urine collections were made for each rat. The rats were not given food while in the metabolism cages but distilled water was always available. The urine was collected in 2 ml of 1 N sulfuric acid. At the end of the collection period, the glass funnel was washed down with distilled water and the total volume made up to 50 ml. Duplicate aliquots were taken for pyruvic acid and thiamine analyses. The

method of Friedemann and Haugen ('43), with slight modification, was used for the pyruvic acid analysis. The thiamine was determined by the method of Mickelsen, Condiff and Keys ('45).

Tissue analysis. At the end of the experiment, the animals were killed by decapitation. The livers of the rats were removed without delay, blotted free of any adhering blood, weighed, and dropped immediately into *0.1 N* sulfuric acid. Analysis for thiamine was made by the thiochrome method, including the enzyme digestion and zeolite adsorption procedure as described by the Association of Vitamin Chemists ('47). The hearts, kidneys, and gastrocnemius muscles of the rats were removed, blotted free of blood, weighed, placed in *0.1 N* H_2SO_4 and analyzed for thiamine. Because of the low thiamine potency of these tissues, and because of their relatively small size, it was necessary to pool the individual tissues of the same kind for each group.

The data obtained on the liver storage of thiamine, the excretion of thiamine and pyruvic acid, and the weight gains were treated statistically, using the "t" test for paired samples as described by Snedecor ('46).

Results and discussion. Mean weight gains, caloric intakes, urinary thiamine and pyruvic acid excretions, and liver storage of thiamine are shown in table 2.

At the end of the experimental period the rats were all normal and healthy in appearance. The average total daily consumption of calories was 2,401. Approximately 570 of these calories were supplied by alcohol to each rat in group 1. The rats fed alcohol gained an average of 93 gm for the experimental period, which did not differ significantly from the average gain of 98 gm for the rats in group 2. Although this difference is not statistically significant, it may indicate that a small percentage of the ingested alcohol was lost through the lungs and urine. Delaunois and Casier ('47) stated that not more than 2.87% of the alcohol ingested by guinea pigs was eliminated by the lungs and not more than 5% in the urine.

It is probable that a similar loss of alcohol occurred in this experiment with rats.

The urinary excretion of thiamine and pyruvic acid varied greatly between the individual rats within each group, as well as in the individual differences between the groups as indicated by the standard error of the mean differences. The amounts of thiamine excreted by the rats in groups 1 and 2,

TABLE 2

Series 1. Mean weight gains, food intakes, thiamine in livers, urinary thiamine and pyruvic acid excretions of paired rats on isocaloric rations with and without alcohol for 10-week experimental period

RATION	NO. OF RATS	TOTAL CALORIES EATEN	WEIGHT GAIN	THIAMINE IN LIVER	URINARY EXCRETION	
					Thiamine	Pyruvic acid
			gm	*μg per gm fresh tissue*	*μg per 24 hr.*	*mg per 24 hr.*
Group 1 Basal ration + alcohol	12	2,401	93	2.73	0.54	2.85
Group 2 Basal ration + dextrose	12	2,401	98	2.19	0.63	3.79
Mean difference ± S.E.M.D.			5 ± 4.5	0.54 ± 0.20	0.09 ± 0.06	0.94 ± 0.6
Significance[1] of difference between treatments			—	+	—	—

[1] Differences are significant when the ratio of the mean difference to the standard error of the mean difference is equal to or greater than 2.201 (Snedecor, '46).

0.54 and 0.63 μg per 24 hours, respectively, did not show a statistically significant difference. The average excretion of 2.85 mg of pyruvic acid per 24 hours for the alcohol group was not significantly different from the 3.79 mg per 24 hours for the group receiving dextrose. However, the trend was toward a lower pyruvic acid excretion for the rats fed alcohol, which may indicate that the rats in this group were in a better

nutritional status in respect to thiamine than the rats fed an isocaloric supplement of dextrose.

The amount of thiamine present in the livers of the rats fed alcohol was 2.73 μg per gram of fresh tissue and in the livers of the dextrose group 2.19 μg per gram of fresh tissue. This difference is statistically significant.

Table 3 shows the results of the thiamine analyses of the liver, heart, kidney, and gastrocnemius muscles of the 12 pairs of rats. For purposes of comparison, the corresponding tissues of animals of the same age reared on the laboratory stock

TABLE 3

Thiamine in tissues of rats fed thiamine-low experimental rations and laboratory stock ration

RATION	NUMBER OF RATS	THIAMINE IN FRESH TISSUE			
		Liver	Kidney	Heart	Muscle
		μg/gm	*μg/gm*	*μg/gm*	*μg/gm*
Basal ration and alcohol	12	2.73	0.22	0.42	0.05
Basal ration and dextrose	12	2.19	0.17	0.43	0.03
Laboratory stock ration	6	9.54	7.38	7.92	1.30

breeders' ration have been included in the table. It is interesting to note that depletion of tissue thiamine can become so extensive without the appearance of some outward signs of a deficiency of this vitamin.

The thiamine values for the hearts, kidney, and muscles of the experimental animals could not be subjected to statistical treatment because the thiamine analyses were made on composite samples. Except for the thiamine content of the heart muscle, the tissues of the rats fed alcohol all appeared to be higher in thiamine than the tissues of the rats fed dextrose. Whether the differences found in the thiamine content of the kidneys and muscles in the two groups are real or not is

questionable. However, the difference in the thiamine content of the livers is statistically significant.

The results of the above experiment suggest the conclusion that the metabolism of alcohol does not deplete the tissue thiamine of rats on a suboptimum intake of thiamine to the extent that the metabolism of an isocaloric amount of dextrose depletes them. These results are in accord with reports in the literature that the substitution of alcohol for carbohydrate delays the onset of thiamine deficiency in rats and pigeons.

Series 2

To test the hypothesis that the metabolism of alcohol does not require thiamine, at least in amounts that would be reflected in storage and excretion, the following experiment was conducted.

Twelve litters of three rats each of the same sex and weight were distributed among three groups in such a way that each group contained a rat of the same sex and weight from each litter. The rats in group 1 received the basal ration only; the rats in group 2 received the same amount of basal ration as group 1 and a supplement of 25% alcohol; group 3 received the same amount of basal ration as group 1 and a supplement of dextrose in an amount isocaloric to the alcohol supplement of group 2. The alcohol supplement was fed as described under series 1. Urine collections, urinary thiamine and pyruvic acid analyses, and thiamine analysis of the livers were carried out as described previously for series 1.

Results and discussion. Comparison of the differences in the mean weight gains, caloric intakes, liver stores of thiamine, and urinary excretions of thiamine and pyruvic acid among the three groups of paired rats are presented in table 4.

Group 1, fed the basal ration only, consumed a total of 2,086 cal. per rat, as compared with 2,656 cal. per rat consumed by group 2, fed the alcohol supplement. That a considerable portion of the alcohol supplement was utilized is indicated by the significant increase of 9 gm in the weight of the alcohol group

above that of the group on the basal ration only. That the metabolism of this alcohol supplement did not cause any increased depletion in the liver storage of thiamine is evidenced by the almost identical values of 2.09 and 2.07 μg per gram that were obtained for the two groups of rats. Again, as in

TABLE 4

Series 2. Comparison of mean weight gains, thiamine in livers, urinary thiamine and pyruvic acid excretions in three groups of paired rats; group 1 fed basal ration only, group 2 fed alcohol supplement, group 3 fed dextrose supplement, in a 10-week experimental period

RATION	NUMBER OF RATS	TOTAL CALORIES EATEN	WEIGHT GAIN	THIAMINE IN LIVER	URINARY EXCRETION	
					Thiamine	Pyruvic acid
			gm	*μg per gm fresh tissue*	*μg per 24 hr.*	*mg per 24 hr.*
Group 1 Basal ration only	12	2,086	103	2.09	0.90	1.52
Group 2 Basal ration + alcohol	12	2,656	112	2.07	0.91	1.98
Group 3 Basal ration + dextrose	12	2,656	119	1.71	0.75	2.07
Mean difference ± S.E.M.D.[1]	Groups 1 and 2		9 ± 3.5	0.02 ± 0.16	0.01 ± 0.10	0.46 ± 0.93
	Groups 1 and 3		16 ± 4.4	0.38 ± 0.15	0.15 ± 0.18	0.55 ± 0.34
	Groups 2 and 3		7 ± 4.5	0.36 ± 0.096	0.17 ± 0.076	0.09 ± 0.37

[1] Differences are significant when the ratio of the mean difference to the standard error of the mean difference is equal to or greater than 2.201 (Snedecor, '46).

series 1, the variation in the amounts of thiamine and pyruvic acid excreted by the rats in the same group and the variation in the differences between groups were large. The differences in the amounts of either thiamine or pyruvic acid excreted by the two groups were not statistically significant.

In comparing the results obtained with group 1 (basal ration) and group 3 (basal ration plus dextrose supplement) it can be seen that the rats fed the dextrose supplement gained an average of 16 gm more than the weight gained by the rats fed the basal ration only. The need for thiamine in the metabolism of the additional calories supplied by dextrose is reflected by the decreased thiamine storage in the livers of the rats receiving the dextrose supplement. There was no significant difference between the two groups in the amounts of either the thiamine or pyruvic acid excreted.

Comparison of group 2 (fed alcohol) and group 3 (fed dextrose) shows that the caloric intake was the same and that the weight gain was not statistically significantly different, although the dextrose group showed an additional gain of 7 gm with a large variation between the differences. This difference in weight gains may also indicate that some of the alcohol was lost through the lungs and in the urine as was suggested under series 1. The mean liver storage of thiamine in the dextrose group, 1.71 μg per gram, was significantly less than the 2.07 μg per gram in the livers of the alcohol group. The amount of thiamine, 0.91 μg per 24 hours, excreted by the rats fed alcohol was significantly greater than the amount, 0.74 μg per 24 hours, excreted by the rats fed the dextrose supplement. These results with respect to liver storage and thiamine excretion indicate that the rats receiving alcohol were not in such a severe state of thiamine deficiency as the dextrose-fed rats. However, the excretion of pyruvic acid in the two groups was very nearly the same.

GENERAL DISCUSSION

The liver stores of thiamine in the groups of rats in both series 1 and 2 indicate that the substitution of alcohol for dextrose in the rations of paired rats resulted in a lower depletion of liver thiamine. The data on thiamine and pyruvic acid excretion in these 4 groups of rats did not offer the conclusive evidence that was expected. As was pointed out by Harper and Deuel ('41), pyruvic acid excretion is dependent on the

state of alimentation of the rat and is greater when the rats are given food at the time of urine collection. In the present experiment it was not considered possible for the rats on paired food intakes to have access to food while in the metabolism cages, because it was not possible to recover and weigh accurately the food spilled. Undoubtedly there was great variability in the amount of food in the alimentary tracts of the individual rats. This factor may have been responsible for some of the variation encountered in the results on pyruvic acid excretion.

When alcohol was fed to one group of rats as an additional supplement and the results compared with those obtained with a group fed the basal ration only (groups 1 and 2 in series 2), it was evident that the utilization of the additional calories supplied by the alcohol did not seem to demand an amount of thiamine over and above that needed for utilization of the calories in the basal ration. However, utilization of the same number of additional calories supplied in the form of dextrose (groups 1 and 3 in series 2) caused a significantly greater depletion of the thiamine stores of the liver.

CONCLUSION

Under the conditions of these experiments the data seem to support the view that the utilization of alcohol fed as a supplement to the ration does not involve an increased need for thiamine.

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